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(30° Ciclo)

Tesi sperimentale

Development of a Local and Alternative Ready-to-Use Therapeutic Food (RUTF) suitable for Community-based Management of Severe Acute Malnutrition.

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Il più delle volte, siamo convinti di essere noi a scegliere il destino, quando invece – spessissimo – è lui a scegliere noi. Questo lunghissimo e sacrificatissimo lavoro che stringete tra le mani ne è un lampante esempio: rappresenta come il susseguirsi degli eventi abbia determinato una lucida e ferrea volontà nel portare a compimento un'impresa che sembrava impossibile agli occhi dei più. Ma non ai miei.

Sono passati *tredici anni* da quando ho messo piede nel Dipartimento (allora Facoltà) di Agraria dell'Università Federico II di Napoli. Qualcuno direbbe: troppo tempo per un ciclo completo di studi; "*un'era geologica*". Per me è stata una trasformazione, un'evoluzione, una tribolazione, un calvario, ma anche un esercizio complicatissimo di temperanza.
Non è stato un percorso semplice, non è stata una galoppata, ma un cammino tortuoso, difficile, pieno di insidie e di imprevisti. Ma, nonostante tutto, eccomi qua.
È stato un *mutatis mutandis*, cognitivo ed esistenziale. È accaduto quello che doveva accadere. Perché se non fosse accaduto non sarei stato qui a scrivere, non avrei trovato la mia essenza.

Ai miei nonni che non ci sono più,
Vincenzo e Ciro.
Ai miei genitori.
Alle donne che ho amato, a quelle che amo e a quelle che amerò.
Al dott. Priore, mentore formidabile.
A Giammarco.

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1. Introduction

1.1. Global matter and experimental design

The Sustainable Development Goal (SDG) 2 proposed by United Nations (UNs) and Food and Agriculture Organization of United Nations (FAO) aims to "End hunger, achieve food security and improve nutrition, and promote sustainable agriculture" by 2030. Actually, last two years' data are not very promising with an absolute global number of hungry people of 815 million in 2016, against 2015's 777 million; furthermore, globally, 155 million under 5 years old children are affected by stunting, 52 million of which by wasting. The reason of this bad change in trend is due to an increasing of conflicts and disasters that go off the achievement of SDG 2 (The Lancet, 2017). An effective and useful tool to treat a part of this enormous problem is represented by the commercial **Ready-to-Use Therapeutic Food (RUTF)**, commonly called **Plumpy' Nut ®**, a pastry spread to squeeze directly in the mouth of under 5 years old children affected by Severe Acute Malnutrition (SAM)**.** Although its great energy and nutritional density, with a lot of effectiveness' studies that show good children recovery rates, this product is often expensive, with costly ingredients and few global suppliers. For these reasons, an increasing number of scholars is focused on the development of ***alternative*** RUTF formulas, taking into account the employment of cheaper ingredients and production processes (Brixi, 2018).

As will be cleared in the following pages, this work regards the development of a process for the production of an alternative and sustainable RUTF (**NutriMax**) for the management of Severe Acute Malnutrition (SAM) in under 5 years old children in Low-Income countries, with the adoption of a less theoretical way compared to other approaches based on the linear programming (Brixi, 2018).

Usually, linear programming uses an optimization formulae algorithm without the testing of some important technological response values, at the beginning of the design. In this case, instead, the first research's target was the selection of ***4 optimized formulas*** (from 20 hypothesized formulas) on the basis of Particle Size Distribution (PSD), rheological and technological (a_w, color, UR%) characteristics. Then, these optimized creams were analyzed under the ***nutritional and sensorial points of view***, to individuate the optimal one to propose as a prototype for further, local, acceptability and clinical trials.

In particular, this PhD-Thesis was based on *three* main steps of research design (**Figure 1.1.** in the next page):

a) A ***preliminary stud***y (**Chapter 2)** of the literature, for the evaluation of technological, nutritional and safety RUTF requirements, to set the constraints for the formula optimization process;

b) A ***small scale production*** **(Chapter 3)** for the alternative RUTF formula optimization, starting by 20 hypothesized recipes, taking into account the nutritional and technological constraints. At this phase **(Chapter 4)**, it was also carried out a production of the optimized formulas for lipid oxidation analysis, finalized to a preliminary stability evaluation;

c) A ***scaled up production*** **(Chapters 5 and 6)** of the alternative RUTF optimized formulas, to evaluate, respectively, sensorial and nutritional characteristics, with the final aim to individuate the optimal alternative RUTF prototype, suitable for local acceptability and clinical trials.

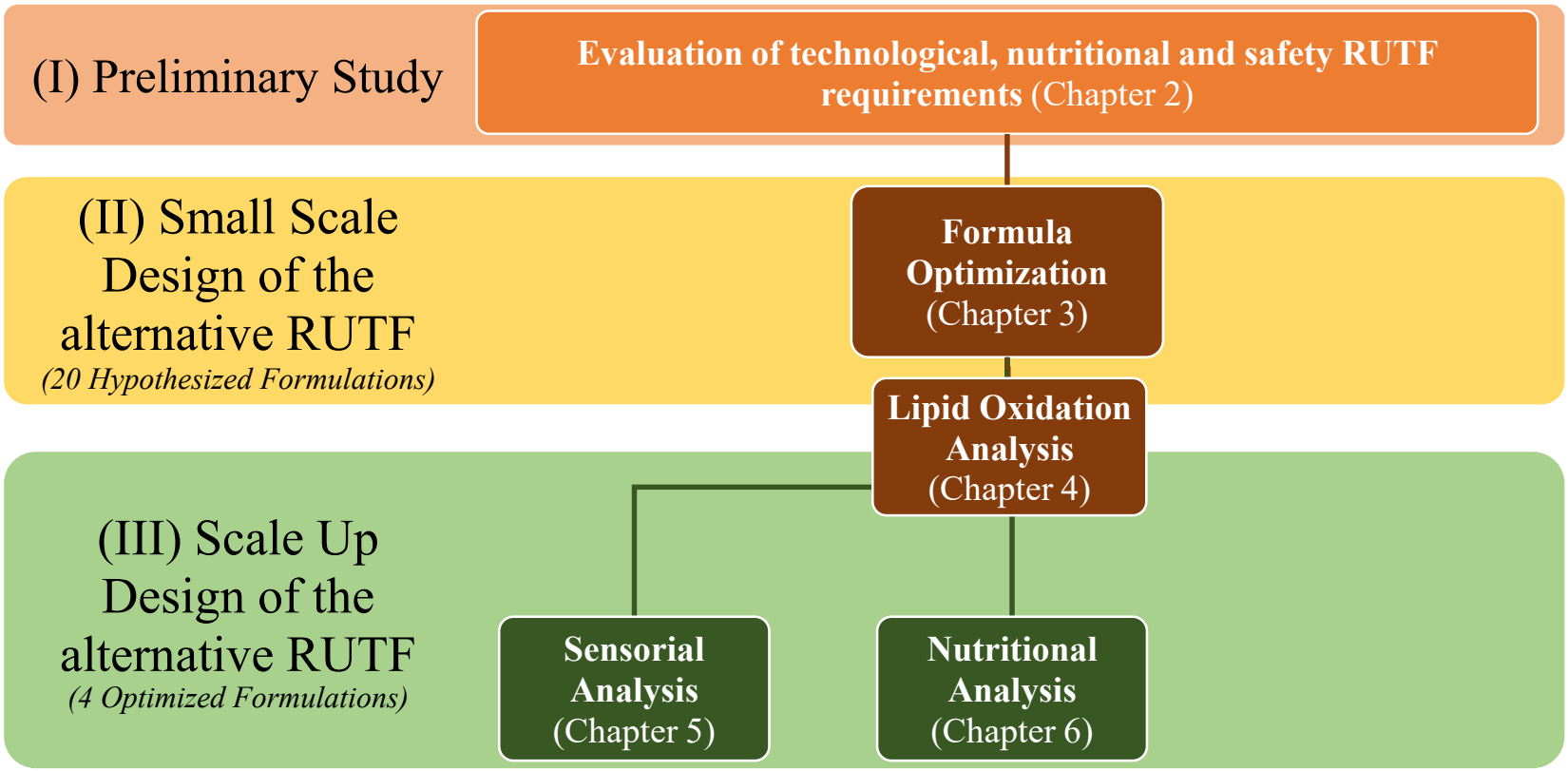

Figure 1.1. Experimental design of the PhD-Thesis.

1.2. Preparation of the alternative Ready-to-Use Therapeutic Food

This paragraph is focused on the description of the alternative RUTF ***production process*** (**Figure 1.2.** in the next page). The details are present in each respective chapter of this thesis.

In general, the employed ingredients for ***small scale production*** were dehulled and roasted soy flour, dehulled and roasted sorghum flour (both for a complete essential amino acid intake), sugar (as simple carbohydrates source), sunflower oil (as fatty acid source), soy lecithin (as emulsifier) and *Arthrospira maxima* (Spirulina) dried powder (for a complete mineral and vitamin intake).

Regarding ***scaled up production***, the ingredients were dehulled soy, dehulled sorghum, sugar, sunflower oil, soy lecithin and *Arthrospira maxima* (Spirulina) dried powder. The treatments, carried out on raw materials, were defined in order to obtain powders suitable for the cream production process. For soy and sorghum, it was necessary to roast, to reduce the water activity and to degrade the anti-nutritional components. For these reasons, the thermal history defined was 120°C for 3 hours in a fluid bed dryer. Subsequently, the grinding step was optimized for both the roasted seeds and the dried spirulina. Raw roasted seeds were initially grinded at 1 mm cut off, then at 250 µm; Spirulina directly at 250 µm. The purpose of scaled up production was to emulate local technological conditions, without availability of transformed ingredients such as flours.

The cream preparation procedure, which consisted of refining and mixing, was performed in planetary ball mills. Prior to begin this operation, it was necessary to define the total quantity of ingredients that had to be used for each processing batch, then the speed and the mixing-refining time of the operation. For ***small scale production***, a laboratory ball mill, with ***250 mL jars***, was employed; on the other hand, for ***scaled up production***, a semi-industrial ***20 kg ball mill*** was used.

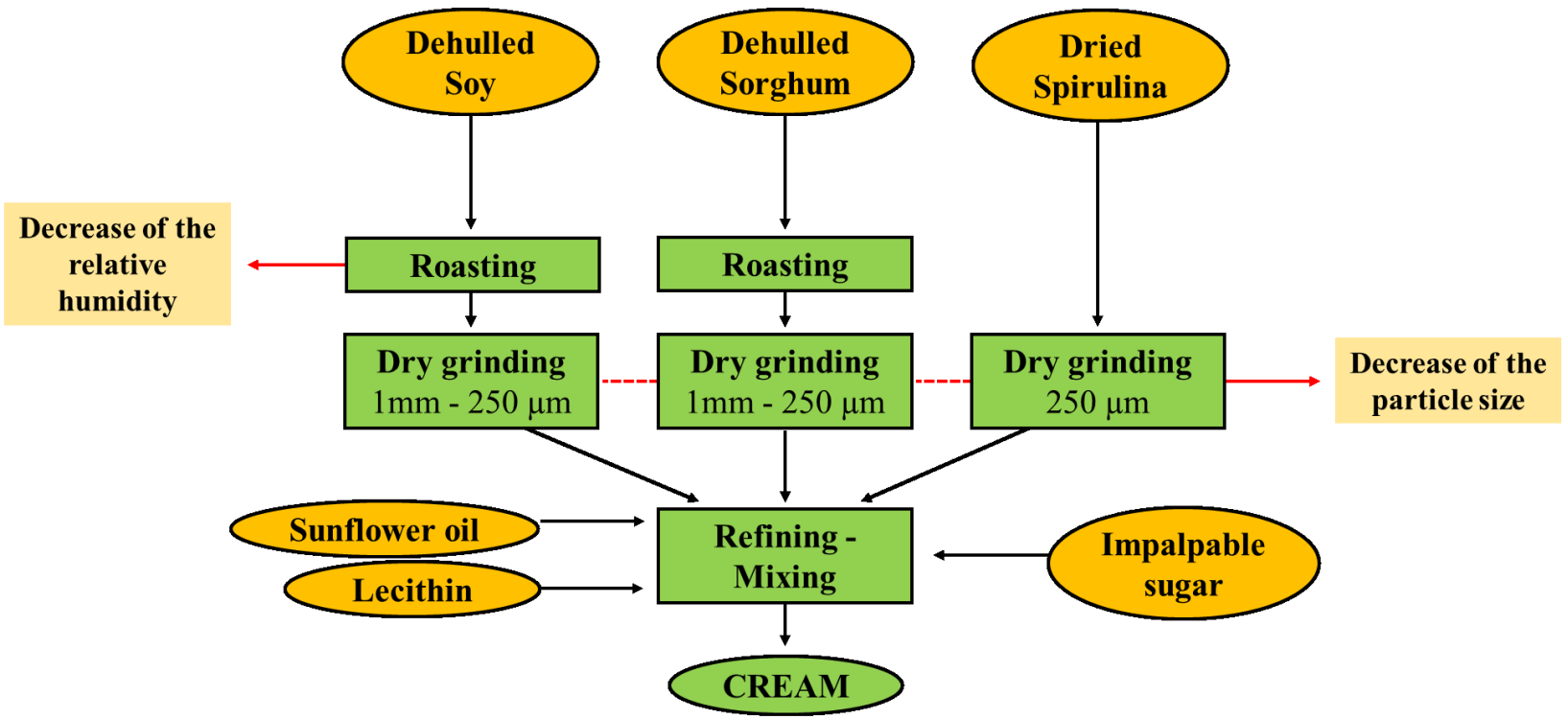

Figure 1.2. Flowchart of the alternative RUTF (NutriMax) cream production process. At *small scale*, the ingredients enter directly in refining phase; at *scaled up* design, the entire scheme can be considered.

1.3. Socio-economical and RUTF production price evaluations

Conflicts all around the world are main cause of food insecurity. In fact, they are a key driver of situations of serious food crisis and famine, especially where wars and social instabilities are prolonged and institutional capacities weak. Watching at Prevalence of Undernourishment (PoU) data, the share of undernourished people in the world decreased from 14.7 percent in 2000 to 10.8 percent in 2013; most worryingly, FAO estimated for 2016 an increasing of PoU to 11 percent. (FAO et al., 2017). To solve this great problem, an approach that aligns actions for immediate humanitarian assistance, long-term development and sustaining peace is necessary.

Sub-Saharan Africa remains he region with the highest PoU, affecting 22.7 percent of the population in 2016. The situation is especially urgent in Eastern Africa, where one-third of the population is estimated to be undernourished – the subregion's PoU increased from 31.1 percent in 2015 to 33.9 percent in 2016 (FAO et al., 2017). Owing in part to the size of its population, the highest number of undernourished people is in Asia.

Children's linear growth in the first five years of life is assessed by the stunting indicator. Stunting is evidence that children are too short for their age, which in turn is a reflection of a chronic state of undernutrition. According to the latest estimates for 2016, 155 million children under five years old of age across the world suffer from stunted growth. At current trends, there would be 130 million stunted children by 2025, which would be 30 million above the global SDG target. Childhood wasting, or being too thin for one's height, reflects a recent and acute process that leads to weight loss and/poor weight gain. In 2016, wasting affected 7.7 percent (51.7 million) of children under five years of age worldwide. About 17 million children suffered from severe wasting (FAO et al., 2017).

Anemia occurs when diet is low in micronutrient content, acute and/or chronic infections, other chronic diseases and cancer, or inherited genetic disorders that affect hemoglobin synthesis. Anemia is an indicator of both poor nutrition and poor health. Anemia is therefore closely linked to other SDG targets – lowering its prevalence will help to reduce maternal mortality and improve levels of economic productivity. The most recent estimates for 2016 indicate that anemia affects 33 percent of women of reproductive age globally. In Africa and Asia, the prevalence is highest at over 35 percent (FAO et al., 2017).

Improved rates of breastfeeding directly contribute to ending hunger and child malnutrition, and increasing the rate of exclusive breastfeeding by up to 50 percent in the first six months of life is one of the global nutrition targets endorsed by FAO. Globally, 43 percent of infants younger than six months were exclusively breastfed in 2016, up from 36 percent in 2005. Between 2005 and 2015, the practice of exclusive breastfeeding increased by at least 10 percentage points in 36 out of 82 countries for which comparable data were available (Horta et al., 2015).

Conflicts affect the food security and nutrition, and lead to a deterioration in food security conditions that can exacerbate, consequently, the conflicts themselves.

FAO classifies 19 countries with a protracted crisis. All of them are also currently affected by conflict and violence, which is typically compounded by adverse climatic events, such as prolonged droughts, that severely affect food production and livelihoods (FAO et al., 2017).

In 2016, more than 2 billion people were living in countries affected by conflict, violence, and fragility. When the state, socio-economic systems and/or local communities do not have the capacities to prevent, cope with or manage situations of conflict, the worst affected are generally the poorest and most vulnerable sectors of society (World Bank, 2017).

People living in countries affected by conflict are more likely to be food insecure and undernourished. The latest FAO estimates for 2016 indicate that 815 million people in the world are undernourished. The majority of these (489 million) live in countries struggling with conflict, violence and fragility (OECD, 2016).

The 2030 Agenda for Sustainable Development makes an explicit link between sustainable development and peace and calls for a transformative approach, with improved collaboration on conflict prevention, mitigation, resolution and recovery. SDG 16 specifically aims to significantly reduce all forms of violence. SDGs 1 and 2 focus on the eradication of extreme poverty and hunger. Achieving these goals is critical for achieving SDG 16 and ensuring peaceful and inclisive societies, leaving no one behind. Correspondingly, achieving SDG 16 will be crucial to meeting SDGs 1 and 2 (FAO et al., 2017).

Although the frequency of wars had been decreasing in recent decades to reach an all-time low in 2005, there has recently been a surge in the number of violent conflicts and conflict-related deaths (IEP, 2016).

Civil wars or internal conflicts have now surpassed the number of interstate or external conflicts between states. There has been a shift from conflict between nations to conflicts within nations. In 1991, internationalized internal conflicts amounted to just 3 percent of total conflicts, a number that soared to 32.5 percent in 2014. As internal conflicts become more prominent, external parties are increasingly likely to become involved or to suffer the consequences of violence; thus, local conflicts evolve into regional or even continental crises (IEP, 2016).

Violence and conflict are unevenly distributed across continents, with most concentrated in four regions: The Near East and North Africa, northern sub-Saharan Africa, Central America, and Eastern Europe, particularly. Many of the most protracted conflicts flow across borders and are regional in nature, including in the Horn of Africa, the Great Lakes region of Africa, between

Afghanistan, India and Pakistan, and from Cameroon, Chad and northern Nigeria across the Sahel (Raleigh et al., 2010).

Where conflict persist over long periods, livelihoods, food systems and resilience become dangerously undermined, creating a downward spiral that results in extended and severe food and nutrition crises (FAO et al., 2017).

Simple correlations show higher levels of chronic and acute food insecurity and undernutrition in countries affected by conflict. In 2016, the unweighted average of prevalence of undernourishment in countries affected by conflict was almost eight percentage points higher than countries not affected by conflict. Almost 22 million, or 75 percent, of stunted children under age five live in countries affected by conflict, with the difference in average prevalence between conflict and non-conflict affected countries at nine percentage points (FAO et al., 2017).

The concurrence of conflict and climate-related natural disasters is likely to increase with climate change, as climate change not only magnifies problems of food insecurity and nutrition, but can also contribute to a further downward spiral into conflict, protracted crisis and continued fragility. The degree to which conflict leads to acute and chronic food insecurity is largely determined by how it affects the lives and livelihoods of individuals, households and communities at different times, together with how it affects the immediate and underlying determinants of individual and household food security and nutrition. Conflict can also have devastating negative impacts on food consumption and health (morbidity and mortality patterns), including the four dimensions of food insecurity (*availability, access, utilization and stability*), individual caring practices, health services and a healthy living environment (FAO et al., 2017).

Conflict impacts can be direct (such as, forced population movements, the destruction of food stocks and productive assets and increased health complications including death) and/or indirect (for example, economic, social

and institutional changes). Indirect impacts can also include disruptions to food systems and markets, leading to increased food prices or decreased household purchasing power, or access to water and fuel for cooking can be reduced, which negatively affects food preparation, feeding practices and food allocation within the household (Justino, 2012).

Conflict and civil insecurity can wreak havoc on economic production and growth, which is detrimental to food security and nutrition not only in that it challenges the availability of and access to food, but also because it presents difficulties in terms of health and nutrition. Conflict duration and intensity are important determinants of economic impact. A recent study that analyzed annual data for 179 countries from 1970 to 2014 not only found that conflict significantly impacted economic growth, but also that the impact increased with the intensity and duration of the conflict (Rother et al., 2016).

Conflict-induced economic contractions reduce employment and income opportunities, which in turn can increase poverty and reduce the ability of households to meet their food and health-care needs. Poverty rates in countries affected by repeated cycles of violence in the last three decades are on average 20 percentage points higher than in non-conflict countries. Conflict can also disrupt export channels and drain foreign exchange resources, limiting import capacity and causing shortages of commodity supplies and inflationary pressure. Import disruptions can lead to reduced food availability in markets as well as reduced availability or affordability of non-food items necessary for food preparation (such as cooking fuel) (World Bank, 2011).

On average, 56 percent of the population in countries affected by conflict live in rural areas, where livelihoods largely depend on agriculture. Most conflicts mainly affect rural areas and their populations, heavily and negatively affecting agriculture, food systems and livelihoods. In many countries affected by conflict, subsistence agriculture is still central to food security for much of the population. Conflict negatively affects almost every aspect of agriculture and

food systems, from production, harvesting, processing and transport to input supply, financing, and marketing. These impacts can be direct and indirect, and felt immediately as well as in the longer-term. Direct impacts can be significant Can be significant, particularly in regard to the destruction of agricultural assets, the forced or corrupt seizure of natural resources, and displacement from land, livestock grazing areas, and fishing grounds. Indirect impacts include macroeconomic shock (FAO et al., 2017).

When conflict and civil insecurity severely disrupt and restrict trade and movements of goods and services, there can also be a negative effect on the availability of food and upward pressure on prices of traded goods, which negatively affects food access. People's physical security is also affected by conflict as it prevents consumers from going to the market or traders from selling their wares. Conflict may also compromise food storage, as facilities can become unsafe or at risk of destruction or looting. However, new market structures can also evolve. The absence of functioning government institutions provides fertile ground for informal markets to flourish, but there is the risk of some groups gaining huge benefits at the expense of others (Simmons, 2013).

In general, to introduce the concept, ***Resilience*** is an important element for coping with conflict and ensuring that shocks and stressors do not have long-lasting consequences for food security and nutrition. Resilience is generally agreed to be a combination of three capacities: *adaptive* (such as risk management, and savings groups), *absorptive* (use of assets, attitudes/motivation, livelihood, diversification, and human capital), and *transformative* (governance mechanisms, policies/regulations, infrastructure, community networks, and formal nets). These three capacities determine how and the extent to which individuals, households, communities, and institutions are able to cope with and adapt to conflict impacts (FAO, 2017).

People typically first engage in reversible coping strategies with short-term effects, such as making modest dietary adjustments and skipping meals.

However, as coping options are exhausted, households are more likely to employ more extreme and damaging strategies that are less reversible and therefore represent a more severe form of coping, such as distress selling of livestock or productive assets such as farm tools. Severe and/or persistent conflict can ultimately lead to the collapse of coping mechanisms, prompting migration, destitution and, in extreme cases, death and starvation. Common strategies to cope food insecurity include: diversification of land holdings and crop cultivation, storage of grain from one year to the next, resorting to sales of assets such as cattle and land that could have been accumulated as a precaution against the occurrence of a shock, borrowing from village lenders or other money loaners, and receiving gifts and transfers from informal mutual support networks (Wood, 2003; Steele, 2007; Justino, 2009).

Building resilience by promoting sustainable peace is critical to improving food security and nutrition outcomes in areas with recurrent crises. First, interventions to improve food security could help weaken some of the causes of conflict, including motives that may lead individuals to support or join armed groups or engage in illegal activities. Second, greater food price stability and the recovery of local agricultural and food markets could help vulnerable individuals and households mitigate the impacts of conflict, including by supporting people affected by conflict in regaining access to markets. As agriculture is the dominant form of livelihood for the majority of households in countries affected by conflict, efforts to revive the sector, foster economic growth, increase food security and improve the nutritional status of the population may also have positive effects on sustaining peace. It is important to rapidly re-engage smallholder farmers in productive activities in the aftermath of shocks, particularly in fragile settings. Social protection, including in-kind and cash assistance, can offer valuable peace dividends and contribute to restoring trust in government and rebuilding social capital (Brinkman and Hendrix, 2011; Kurtz and McMahon, 2015).

There are a number of food security and nutrition-related interventions and measures that can be put in place to prevent and mitigate the risk of conflicts recurring, for example, through price stabilization measures and social protection interventions. A different approach is to drive recovery through agriculture, thereby bringing new life to shattered homes and communities, and motivating people to come together after a conflict has destroyed social networks. The international community should pay special attention to post-conflict situations when seeking to sustain peace. However, there is increasing recognition that sustaining peace is no longer just a post-conflict activity, but should be a priority during all stages of the conflict cycle – before, during and after (Parker et al., 2013).

In the face of these matters, alternative RUTFs can be considered a valid tool to buffer the child undernutrition in a sustainable and innovative way, directly in place.

To enforce households' resilience in the management of under five-years old severe malnutrition, the commercial RUTF, the **Plumpy' Nut ®** was introduced. As we also wrote before, Plumpy' Nut ®, is affected by the high cost problem, because of license fee and also of some expensive ingredients, such as skimmed milk powder, peanuts and palm oil, that account for over 50% of product mass and for most of the total ingredient cost (Santini et al., 2013; Brixi, 2018). Our formulations, instead, present a sensible lower ingredients' prices as shown in **Figure 1.3.** (in the next page). Comparing the key ingredients prices of Plumpy' Nut ® (milk, peanuts, palm oil and sunflower oil) and those of our alternative RUTF **NutriMax** optimized preparations (sorghum, soybeans, sunflower oil), on the World and Africa markets, it's clear that the impact on the global production costs is more dramatic on Plumpy' Nut ® than on NutriMax.

Furthermore, taking into account the actual World market price of **skimmed milk powder** – the real dairy ingredient used in Plumpy' Nut ® preparation –

in the order of more of ***1,900 USD/ton***, the bad economic role of this particular element on final RUTF cost is clear, so the hypothesis of milk replacement in the alternative formula can be considered as a key way to low final aggregate costs.

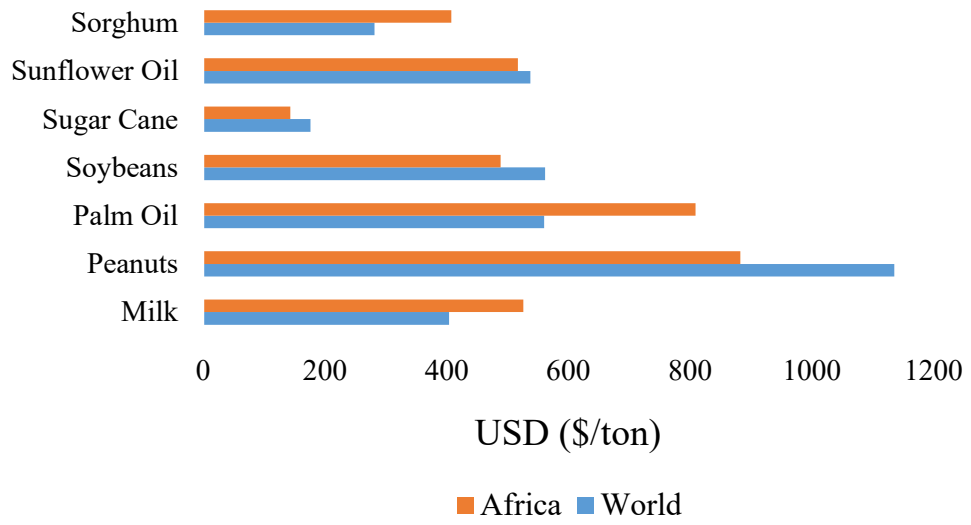

Figure 1.3. Comparison of the key ingredient prices of the *Plumpy' Nut* ® (Milk, Peanuts, Palm Oil and Sunflower Oil) and those of our alternative RUTF – *NutriMax* (Sorghum, Sunflower Oil and Soybeans), taking into account the USD/ton producers prices on Africa and World market. Raw data source: FAOSTAT, 2018.

In **Figure 1.4.** (in the next page) is shown a comparison among the summarized key ingredient costs (on the African market) of the alternative RUTF NutriMax optimized formulations and of the Plumpy' Nut ®, normalized for each recipe. Plumpy' Nut ® is sensibly more expensive than NutriMax optimized formulations, under the profile of ingredient costs.

Regarding Spirulina, taking into account the very few amount of the dried biomass to add into formulation, our idea is to produce this ingredient directly in place, without a costly purchasing of already done biomass, with a very positive impact on the economic sustainability of the process. Furthermore, considering the complete replacement of the complex of minerals and

vitamins, used for Plumpy' Nut ® (**§ Chapter 2**), with Spirulina dried biomass, the economic advantage is more effective and clear. Fortunately, cultivation conditions of this microorganism are simple to manage and are also cheaper than other microalgae, or Generally Recognized As Safe (GRAS) synthetic or natural ingredients.

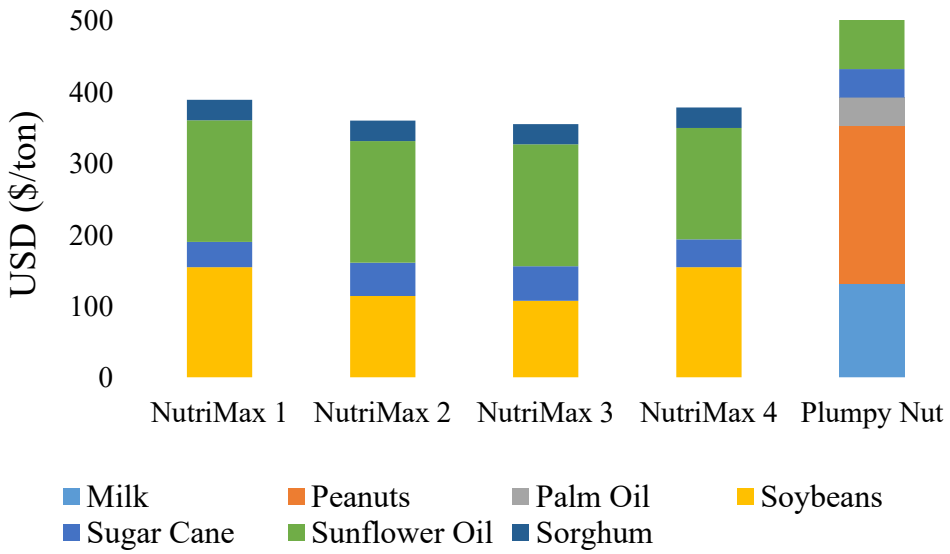

Figure 1.4. USD/ton prices (on the African market) of the 4 optimized alternative RUTF formulas (*NutriMax 1, 2, 3, 4*) and of the *Plumy Nut*, taking into account the specific recipes and the only key ingredients. Raw data source: FAOSTAT, 2018.

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2. Preliminary Study: evaluation of technological, nutritional and safety RUTF requirements

2.1. Severe Acute Malnutrition

Wasting affects about 51 million children, while severe wasting almost 17 million of them (FAO et al., 2017). Moreover, it is estimated that 5.9 million children under five years old die each year and half of them due to Severe Acute Malnutrition (SAM) (UNICEF, 2016).

2.1.1. Clinical manifestations of SAM

SAM is a clinical framework that could have several expressions: marasmus, kwashiorkor and marasmic kwashiorkor. It's very important to carry out a fast diagnosis, to provide an adequate treatment, often directly at home with a Community-based Management of Acute Malnutrition (CMAM), depending on the presence or less of complications, such as chronic or acute infections or illnesses. In this context, is very important to choose a good anthropometric way for health evaluation of each children; the most used one is Mid-Upper Arm Circumference (MUAC), with a good provisional diagnosis effectiveness in literature (Ashworth, 2006).

2.1.1.1. Marasmus

Children affected by marasmus are weak and emaciated, with important weight loss in a relative brief period. Two anthropometric ways are admitted for marasmus diagnosis: MUAC < 115 mm, or Weight-for-Height Z-score (WHZ) more than three SDs below the mean (WHO, 2009). The optimum degree of precision in marasmus diagnosis is reached using both MUAC and WHZ, but

in rural regions, where the resources are limited, the only MUAC is admitted, in order to simplify the work and to permit the diagnosis also by laypeople (Trehan and Manary, 2015).

2.1.1.2. Kwashiorkor

Kwashiorkor is another form of SAM, characterized by a symmetric bilateral pitting oedema that begins in the feet (1+ oedema), then the lower legs and hands (2+ oedema) and in most severe cases can involve the face (3+ oedema) (Ahmed et al., 2009). Often, kwashiorkor is the result of protein deficiencies due to an inadequate nutritional intake.

2.1.1.3. Marasmic kwashiorkor

Marasmic kwashiorkor is a specific clinical framework characterized by both marasmus and kwashiorkor. Children affected by this form of SAM are usually the worst ones, because they are with the highest risk of mortality (Trehan and Manary, 2015).

2.1.2. Treatment of SAM

To assess an effective treatment of SAM, is important to carry out the identification and diagnosis of malnourished children. Until some years ago, only inpatient hospital care was provided, while since early 2000s a new community-based approach has been possible, thanks to introduction of Ready-to-Use Therapeutic Foods (RUTFs). Children are preliminary evaluated on the basis of anthropometric parameters and clinical health complications as infections or illnesses. If it's not necessary a hospital stabilization, a

community-based care at home can be carried out, using a RUTF feeding for 10 – 12 weeks.

2.1.2.1. Preliminary evaluations

First of all, children are evaluated about the presence of marasmus, kwashiorkor or both of them, following the anthropometric parameters previously explained. If children are affected by SAM, a clinical complication evaluation starts, putting attention on anorexia, dehydration, high fever, respiratory distress, hypoglycemia. When clinical complications are not significant, the outpatient treatment can start directly at home, in the village; instead, when illnesses are dangerous for children, the inpatient treatment in hospital is considered as mandatory.

2.1.2.2. Outpatient approach (CMAM)

Decentralized outpatient treatment, following a CMAM model, was a significant evolution of traditional hospital management of SAM. CMAM is very important to increase the treatment effectiveness of SAM, thanks to several factors, such as the nearness of children to their siblings and parents. Furthermore, mothers can effectively care all children of the family, because are not forced to follow their sons in the hospital, leaving the home.
Children evaluated as malnourished start with a test feeding of 30 g of RUTF. If they are able to swallow, they are discharged home with a 1 week to 2 week supply of RUTF, setting a dose of 200 kcal · kg body weight^{-1} · day^{-1} (Santini et al., 2013). Every 1-2 weeks, children have to be re-evaluated, until no more oedema, or WHZ > -2 SD, or MUAC > 125 mm. Further interventions are admitted in a CMAM model, when children are able to stay at home. For

instance, they can be treated by vitamin A, or folic acid supplementation, antimalarial medication and routine antibiotics (WHO, 2013).

2.1.2.3. Inpatient approach

When outpatient approach is not possible because of prohibitive health conditions, only solution is SAM inpatient therapy. Inpatient therapy consists in a coordinated 10-step protocol, providing treatments for hypoglycemia, hypothermia, dehydration, electrolyte imbalance, infections, micronutrient deficiencies, feeding deficiencies, growing-up problems and sensory alterations (Ashworth et al., 2003). During this protocol, two types of therapeutic milk are administered: F-75 formula (75 kcal ·100 ml^{-1}) and F-100 formula (100 kcal ·100 ml^{-1}). The former is used during the first part of treatment, while the latter in the final one, when children feel better. Differences in energy, osmolarity, protein and micronutrient concentration characterize these milks (**Figure 2.1.**).

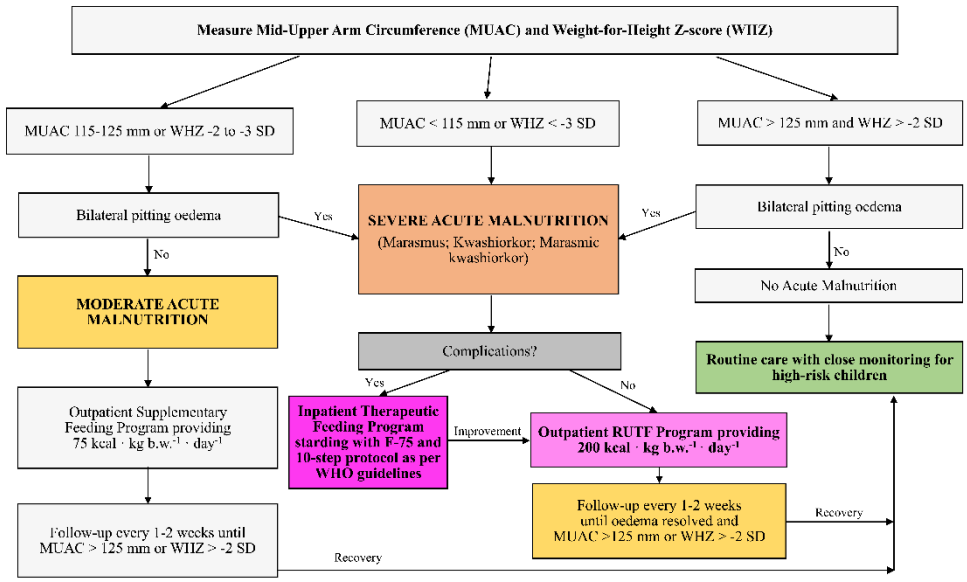

Figure 2.1. Treatment algorithm for acute malnutrition in children.
Modified from Trehan, I., and Manary, M.J. (2015). Management of severe acute malnutrition in low-income and middle-income countries. Archives of Disease in Childhood 100, p. 285.

2.2. Ready-to-Use Therapeutic Food

RUTF revolutionized SAM treatment, thanks to the introduction of CMAM model, with very encouraging results in recovering from SAM complications. Until RUTF introduction, SAM treatment was based on F-75 and F-100 milks, as already explained. In particular, RUTF derived directly from F-100 nutritional intake, with deeply different technological characteristics.

F-100 is a therapeutic milk that needs to be reconstituted with clean water and heated before use and, if left unrefrigerated, the solution spoils. Therefore, this food has to be managed necessary in inpatient therapy, with well-prepared personnel. Surely, F-100 is not suitable for outpatient treatment of SAM. To find a food good for CMAM model, Michel Lescanne, the director of Nutriset, a French private company founded in 1986 specializing in therapeutic foods, and André Briend, a French scientist of the Institute of Research for Development (IRD), in the mid 1990s tried several forms of RUTF, with similar F-100 composition (Guimon J. and Guimon P., 2012). After some attempts, they came up with a viable option based on peanut butter, skimmed milk powder, vitamins and mineral. The concept was to minimize water content, adopting a paste very rich in lipids and proteins, such as the most famous squeezable chocolate creams. The name chosen to identify the product was *Plumpy' Nut* ®, a melting of the words "plumpy" and "peanut" (**Figure 2.2.**).

Figure 2.2. Primary packaging sachet of Plumpy' Nut ®.

At the end, large-scale production of Plumpy' Nut ® came up in the late 1990s at Nutriset's factory in Malaunay, France. Nutriset and IRD started with some clinical trials in Africa and, contemporary, registered the product and the production process in the French patent office in 2000 (patent number 00/13731), then in the US Patent and Trademark Office also in 2000 (patent number 6,346,284) and in the European Patent Office in 2001 (patent number AP/1647) (Guimon J. and Guimon P., 2012). Patent had the objective to standardize the product process and characteristics for a better impact on the quality of treatment and the acceptability by malnourished children. Actually, at that period, demand was not so high, because of difficult exportations of the product from France to low-income countries. A significant increase of demand was after the early successful international clinical trials and, mostly, after the introducing of a more efficient distribution program called PlumpyField network in 2005. The aim was to empower the diffusion of a local production, with the shape of franchising, through an easy Patent Usage Agreement to purchase. Some local producers started their activity, but only thanks to a facilitation in license access in 2010 (patent usage fee set at 1% of the turnover earned by the sale of finished products) and to the intervention of Governative Organizations (GOs) and Non Governative Organizations (NGOs) and as official purchasers, the distribution net increased its effectiveness and the demand grew up. In any case, local production sustainability is still far to reach good levels of diffusion, due to distribution, purchasing and ingredient cost issues (especially skimmed milk powder), so new alternative RUTF recipes would be designed and carried out.

2.2.1. Specifications of a RUTF

Commercial RUTF (commonly known as Plumpy' Nut ® and produced by Nutriset – France) is a squeezable pastry food, made by peanuts, icing sugar, rapeseed and palm oil, skimmed milk powder and a mix of vitamins and minerals (**Table 2.1.**).

Table 2.1. Commercial RUTF ingredients.

Ingredient	Percentage (%)
Peanut butter	25.0
Vegetable oil (rapeseed oil + palm oil)	20.0
Skimmed milk powder	25.0
Icing sugar	28.0
Mineral and vitamin mix	2.0
Total	100.0

Data from Fellows, P. (2012). Local production of ready-to-use therapeutic food for the treatment of severe acute malnutrition. Food Chain 2:2, p. 189.

Under specific package conditions, with a nitrogen atmosphere, the shelf life of the product is until 24 months, at room temperature. This result is possible thanks to a very low level of water, due to an almost anhydrous matter. The nutritional specifications were informally established by World Health Organization (WHO) (2007), then amended by United Nations International Children's Emergency Fund (UNICEF) - Medecins Sans Frontieres (MSF) conferences in 2012 and 2013 (**Table 2.2.** in the next page).

Table 2.2. Commercial RUTF composition

Component	Amount (per 100 g)
Moisture (g)	< 2.5
Water activity – a_w	< 0.6
Energy (kcal)	520-550
Proteins (g)	12.8-16.2
Lipids (g)	25.8-36.3
Fibres (g)	< 5
Sodium (mg)	290
Potassium (mg)	1100-1400
Calcium (mg)	300-600
Phosphorous [a] (mg)	300-600
Magnesium (mg)	80-140
Iron (mg)	10-14
Zinc (mg)	11-14
Copper (mg)	1.4-1.8
Selenium (mcg)	20-40
Iodine (mcg)	70-140
Vitamin A (mg RE)	0.8-1.2
Vitamin D (mcg)	15-20
Vitamin E (mg)	> 20
Vitamin K (mcg)	15-30
Vitamin B1 (thiamine) (mg)	> 0.5
Vitamin B2 (riboflavin) (mg)	> 1.6
Vitamin C (mg)	> 50
Vitamin B6 (mg)	> 0.6
Vitamin B12 (mcg)	> 1.6
Vitamin B9 (folic acid) (mcg)	> 200
Vitamin B3 (niacin) (mg)	> 5
Vitamin B5 (pantothenic acid) (mg)	> 3
Vitamin B7 (biotin) (mcg)	> 60

[a] Expressed in terms of non-phytate phosphorous.

Data from WHO (2007). Community-based management of severe acute malnutrition. World Health Organization, World Food Programme, United Nations System Standing Committee on Nutrition, United Nations Children's Fund; UNICEF, MSF (2012). RUTF product specifications. United Nations Children's Fund, RUTF pre-bid conference, August 15th 2012. Available at https://www.unicef.org/supply/files/5._Common_RUTF_Specifications_and_Labelling.pdf Accessed on March 15th 2018;

UNICEF, MSF (2013). RUTF product specifications. United Nations Children's Fund, RUTF pre-bid conference, September 12th 2013. Available at https://www.unicef.org/supply/files/Odile_Caron_RUTF_Product_Specifications.pdf Accessed on March 15th 2018.

Regarding raw materials for RUTF production, milk shall be in powder form, following:

a) Codex STAN 207-1999: Codex Standard for Milk Powders and Cream Powder;
b) Codex STAN 289-1995: Codex Standard for Whey Powders.

Peanut or peanut paste shall follow the prescriptions of:

a) Codex STAN 200-1995: Codex Standard for Peanuts;
b) CAC/RCP 55-2004: Code of Practice for the Prevention and Reduction of Aflatoxin Contamination in Peanuts.

Lipidic phase shall be rapeseed oil, or sunflower oil, both mixed with palm oil (the last added to increase the technological properties of the paste). Normative reference is:

a) Codex STAN 210-1999: Codex Standard for Named Vegetable Oils.

Carbohydrates usually used in RUTF formulations are lactose, sucrose, maltodextrine, fructose and starches. Normative reference is:

a) Codex STAN 212-1999: Codex Standard for Sugars.

Complex of minerals and vitamins shall provide from the list of sources of premix authorized by World Food Programme (WFP) and its composition shall not alter the acid-base metabolism of patients.

Emulsifying agents are very important to slow the phase separation of the formulation. It is suggested the use of lecithin (max 0.5 g · 100 g^{-1}), or mono and diglycerides (max 2 g · 100 g^{-1}).

Artificial flavourings are not allowed. Admitted antioxidants are ascorbyl palmitate and mixed tocopherols.

An official Governative standard doesn't exist. A draft of Guideline for RUTFs was deposited at Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) of Codex Alimentarius Commission (CAC), supervisioned by Food and Agriculture Organization of the United Nations (FAO) and WHO (CCNFSDU (2016)) and will be discussed, improved and amended next years,

with the purpose to finalize the process e to approve the document by 2020. At the moment, each producer is suggested to follow the WHO and UNICEF informal specifications and the Good Manufacturing Practices (GMPs) for high lipid foods.

2.2.2. Production of a RUTF

Technologically, RUTF is characterized by water-soluble elements, mixed with a lipid phase. To achieve a good homogeneity of the paste, specific order of passages has to be followed (**Figure 2.3.**). In particular, as first step, the lipid phase is stirred and slightly heated, then the powdered ingredients are slowly added, while a strong stirring is carried out. Finally, a last stirring with both solid and lipid elements together is carried out, before packaging into full nitrogen atmosphere in plastic-aluminum primary bag. (Manary, 2006).

Figure 2.3. Gross RUTF production scheme.

2.2.3. Local production of a RUTF

Local equipment for RUTF-making depends on the scale of production. In any case, a set of stainless steel or food grade plastic containers, a scales, a mix, a filler and a heat sealer are always required.

Small-scale production (< 500 kg each week) requires a planetary mixer to blend lipidic phase with dried powders in a batch manner. It's very important to blend the RUTF paste before slowly, then stronger, to ensure a homogeneous matter and to prevent the oil syneresis (phase separation). Final

RUTF can be put into 250 g bottles, a typical daily dose for a child affected by SAM.

At medium-scale production (> 500 kg and < 1,500 kg each week), another type of machine is used, in order to mix, to grind and to fill a container also, automatically. Usually, these facilities are provided by pastry factories.

At large-scale production (> 3000 kg each week), automatic flow devices are used, in order to ensure a continuous flux of material from initial ingredients to final enclosed product (Manary, 2006). In particular, a couple of machines are connected: usually, a horizontal helical mixer and a horizontal form-fill-seal machine.

It's strongly important to respect a logical flow of materials in the environment of work, to avoid the cross contamination (Fellows, 2012) (**Figure 2.4.**).

Figure 2.4. RUTF logical flow diagram. In yellow, the ingredient steps; in red, the weighing, mixing and filling-sealing steps; in green, final RUTF product storage phases.

2.2.4. Microbiology, Quality Assurance and process validation

At the moment, a unique international microbiological and quality standard for RUTF production and process validation doesn't exist. This is a great problem, because GOs and NGOs suggest some important microbiological criteria and production practices, but each producer can choose by itself.

First of all, in 2015, a Code of Hygienic Practice for Low-Moisture Foods was adopted by Codex Alimentarius Commission. It's an important standardizing quality reference for RUTF producers, in substitution of generic GMPs (CAC, 2015). In second place, there are a lot of unspecific standards that have to be considered by producers for RUTF quality assurance and microbial safety: in particular, Hazard Analysis Critical Control Point (HACCP) principles; Code of Hygienic Practice for Powdered Formulae for Infants and Young Children (CAC, 2009) and ISO 22000:2005 (ISO, 2009).

Microbiological specifications, proposed by UNICEF, MSF, WHO and WFP in various documents, include criteria for Enterobacteriaceae, *Salmonella* spp., *Cronobacter* spp. (a microorganism very dangerous, able to grow into a low moisture matter), mesophilic aerobic bacteria, coliforms, and *Listeria* spp. (**Table 2.3.** in the next page).

Table 2.3. RUTF safety requirements

Sources	Safety requirement
Mesophilic aerobic bacteria	< 10,000 CFU in 1 g
Coliform test	Negative in 1 g
Yeast	< 10 CFU in 1 g
Moulds	< 50 CFU in 1 g
Cronobacter sakazakii	< 10 CFU in 1 g
Clostridium perfringens	Negative in 1 g
Pathogenic staphylococci	Negative in 1 g
Salmonella spp.	Negative in 25 g
Listeria spp.	Negative in 25 g
E.coli	Negative in 1 g
Enterobacteriaceae (30°C)	< 10 CFU in 1 g
Total aflatoxin	< 10 ppb

CFU: Colony Forming Unit
Data from UNICEF, MSF (2013). RUTF product specifications. United Nations Children's Fund, RUTF pre-bid conference, September 12th 2013. Available at https://www.unicef.org/supply/files/Odile_Caron_RUTF_Product_Specifications.pdf Accessed on March 15th 2018.

Application of HACCP principles is important to achieve a safe final product. In fact, a good HACCP principles implementation reflects on a lower risk of aflatoxin contamination, both in the ingredient storage phase and in final RUTF product maintaining. Furthermore, with a HACCP plan, cost-effectiveness of the entire process will improve, ensuring a very high quality final matter (Henry and Xin, 2014).

Applying ISO 22000:2005, then, the traceability of the whole process passages is ensured. The history of the entire process is very fundamental for saving measures in case of health problems during the distribution, or after the consumption of the spread.

A stability study on the final product is mandatory for shelf-life determination, before out of primary packaging, then, after good preliminary results, inside the packing, eventually in presence of modified atmosphere (especially for offshore production). Instead, in the case of local production, a simpler storing technology is suggested, to avoid an unsustainable increasing of the costs. In

general, for local production spoons and plastic bags are enough to distribute and administer the therapeutic paste.
Finally, validation process is ensured following the commonly recognized specifications promoted by international purchasers, GOs and NGOs.

2.2.5. Effectiveness, sustainability, costs and alternative recipes of the RUTF

Effectiveness of RUTF outpatient SAM treatment, compared to standard inpatient one, is underlined by a lot of papers published during last years (Defourny et al., 2007; Eklund and Girma, 2008; Gaboulaud et al., 2007; Navarro-Colorado and Laquiere, 2005).
Despite these results, some relevant problems emerged year by year. As reported before, in a first time, demand didn't increase due to licensing and high production costs, especially skimmed milk powder, but also to acceptability issues by children. Only after the introduction of PlumpyField network in 2005, situation improved a bit and demand grew up, thanks to the raise of some local producers and purchasing/distribution promoted by UNICEF and NGOs. This evidence, however, is not enough to solve in great part the problem, as desired by GOs and NGOs. In fact, UNICEF procured 35,000 metric tons (MT) of RUTF paste in 2015, 33,000 MT in 2016 and 36,000 MT in 2017, in manner to treat 2.5 million malnourished children. Thus, 17 million wasted children are interested by SAM across the world, but only 15% of them is actually treated (UNICEF, 2017 a). Despite UNICEF and other NGOs efforts in supplying low income countries, most of malnourished children are not treated with RUTF paste.
RUTF world suppliers increased form one in 2007 to 23 in September 2016, with 18 of them in countries at high concentration of acute malnutrition. This should reflect on an increasing of efficacy in RUTF treatment covering, but actually is not true. Local production of RUTF is affected by high management

costs linked to license fee, raw materials, mineral and vitamin mix, packaging and final product distribution issues across villages and rural areas. In particular way, skimmed milk powder cost is so much high, especially if imported in low-income countries with high customs duty. So, local production is not a panacea, but need of an optimization in expansion of the suppliers, in brand liberalization, and in logistic management of raw and final product fluxes (Segrè et al., 2017). Furthermore, PlumpyField network and license fee are limits to the raise of a wide larger number of small local producers, with lower distribution costs and a more effectiveness internal management. In fact, almost paradoxically, local RUTF prices are 12 – 14% higher than offshore produced RUTF (UNICEF, 2017 a) (**Figure 2.5**.).

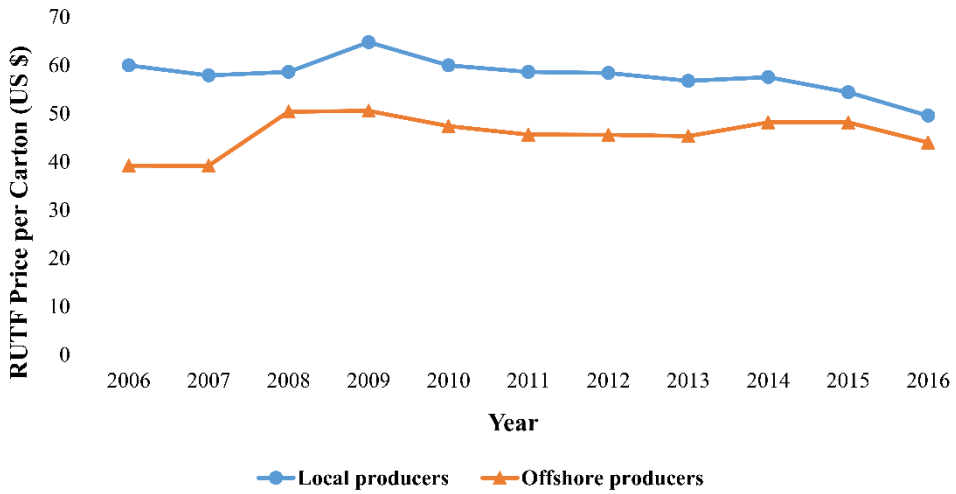

Figure 2.5. Mean in US dollars of local and offshore RUTF suppliers' prices by year, calculated considering a price mean by year of all local and offshore UNICEF suppliers. All prices were converted in US dollars.
Data from UNICEF (2017 b). Ready-to-use therapeutic food price data. UNICEF Supply Division, February 2017. Available at https://www.unicef.org/supply/files/RUTF_Prices.pdf Accessed on December 13th 2017.

As ultimate ideal solution, the implementation of RUTF alternative recipes without skimmed milk powder, and an increasing of the number of RUTF local producers, should significantly low the ingredient and logistic costs, and finally the end product price.

Taking into account the WHO nutritional specifications for RUTF formula and the alternative ingredient quantities of macro and micronutrients, potential alternative recipes for local RUTF are effectively possible. A nutritionally suitable multimix for RUTF has four basic ingredients:

a) A dried cereal as staple ingredient;

b) A dried vegetable or animal protein supplement. To significantly low the ingredient cost, legumes or oilseed is preferable;

c) A dried colored vegetable or fruit as vitamin and mineral supplement;

d) Oil and sugar used as energy supplement to increase the energy concentration of the mix (Collins and Henry, 2004).

Several attempts of alternative RUTF recipes were done last years, with controversial results. The aim was to low production costs, maintaining the same effectiveness of commercial RUTF. Recent clinical trial studies showed that soya-maize-sorghum RUTF (SMS-RUTF) had the same therapeutic recovery effects of commercial RUTF as written by Bahwere et al. (2016). On the other hand, other studies didn't confirm this hypothesis of equivalence between SMS-RUTF and commercial one, as showed by Irena et al. (2015). Furthermore, acceptability of new RUTF products is likewise fundamental to set a right alternative RUTF recipe. Often, studies showed that acceptability of the same product changed by country, or even by local region (Owino et al., 2014; Weber et al., 2017).

In conclusion, further studies are mandatory to set an effective and concrete alternative to commercial RUTF, for a real and sustainable expansion of RUTF supplying, covering the large part of malnourished children still untreated today.

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3. Formula optimization approach for an Alternative Ready-to-Use Therapeutic Food

3.1. Materials and methods

3.1.1. Materials

For RUTF formula optimization, the following ingredients were used: sunflower oil (Grazia, Lucca, Italy) and soy lecithin (ACEF, Piacenza, Italy), as lipidic phase, dehulled and roasted soy flour (Consorzio Agrario dell'Emilia Romagna, Bologna, Italy) and dehulled and roasted sorghum flour (Consorzio Agrario dell'Emilia Romagna, Bologna, Italy), as flour mix, and icing sugar (Eridania, Bologna, Italy). Microalgae Spirulina dried powder was also used (produced in CAISIAL-University of Naples, Portici, Italy). All the powders presented a Particle Size Distribution lower than 250 µm and a moisture content lower than 1%. A commercial RUTF (Plumpy' Nut ®, Nutriset, Malaunay - France) was analysed in order to compare the characteristics of the new formulations with that widely diffused commercial RUTF product.

3.1.2. Experimental design

3.1.2.1. Experimental design

A D-optimal mixture design was used (Miele et al., 2015). Only one ingredient was constant, dried Spirulina powder, representing only 3% of the mixture to ensure a good nutritional intake and, contemporary, to avoid hypervitaminosis. The variable ingredients (97%) consisted of flour (x1), lipidic phase (x2) and icing sugar (x3). The proportions of each ingredient were expressed as a

percentage of the mixture, and for each treatment combination, the sum of the component proportions was equal to one hundred, where:

$$x1 + x2 + x3 = 100 \quad (3.1)$$

Preliminary tests of pastry-mixing workability and specific nutritional requirements for RUTF (Santini et al., 2013) allowed to set the following ranges for each ingredient: flour mix (x1) 30–40%; lipidic phase (x2) 25–35% and icing sugar (x3) 25–35%.

A Cubic model (Gacula, 1993) was used and 20 experimental points were determined, 5 points were replications. In Table 1 Oil/Powder and Oil/Icing Sugar ratios are reported for each formulation instead of the ingredient concentration.

Each formulation was replicated three times.

3.1.2.2. Sample preparation

For cream production, a stirred ball mill was used (model PM 200, Retsch, Haan, Germany), equipped with two jars (250 mL). 5 spheres of 2 cm diameter and 4 spheres of 1 cm diameter were used. Ingredient weight was set on 50 g, according to manual specifications of the instrument. Through preliminary experiments, the optimal refining conditions were determined. Optimal speed rate and optimal time were set using both the higher and lower Oil/Powder ratio creams, measuring the Particle Size Distribution (PSD) at 400, 500 and 600 RPM, every 30 minutes, from zero time, to 180 minutes. In particular, the speed rate that, in the shortest time, gave the thinnest PSD was set (500 RPM; 120 minutes).

3.1.3 Sample characterization

3.1.3.1. Particle size measurement

A Mastersizer laser diffraction particle size analyser equipped with Hydro 3000 dispersion unit (Malvern Instruments, Worcestershire, UK) was used. About 0.1 g of paste was analysed at ambient temperature (20±2°C), as reported in the recent paper of Fidaleo et al. (2017). Several indexes of the PSD based on the volume of particles were estimated, including the D_{10}, D_{50} and D_{90}, respectively.

For each cream two different replicates were analysed and for each replicate 15 measurements were performed.

3.1.3.2. Viscosity measurement

Viscosity of cream samples was determined by a rotational rheometer ARES-LS (Rheometrics Inc., Piscataway, NY). The flow curves were carried out at 30°C using a plate-plate tool adopting the procedure reported by Fidaleo et al. (2017). Three replications for each sample were performed. The rheological behaviour of the cream samples was described with the Ostwald de Waele model and the corresponding model parameters (n and K) were estimated (Glicerina et al., 2013; Fidaleo et al., 2017).

3.1.3.3. Water activity

Water activity (a_w) was evaluated by an Aqualab-Dew point water activity meter (4TE, USA). Three replications for each sample were performed.

3.1.4 Data analysis

One-way analysis of variance (ANOVA) and multiple comparisons (Duncan) were used to evaluate if differences among the sample means were statistically significant ($p \leq 0.05$). The statistical software SPSS for Windows, version 17.0 was used for data analysis (SPSS Inc., Chicago, IL, USA).

As response variables, a_w values, Particle Size Distribution parameters (D_{10}, D_{50}, D_{90}), consistency index (K) and flow index (n) were used. Only the variables significantly different were used in models generated to determine the optimal levels of ingredients. The following regression model was used:

$$Y=B1X1+B2X2+B3X3++B12X1X2+B13X1X3+B23X2X3+B123X1X2X3+error \quad (3.2)$$

where Y was the response variable, X was the percentage of each ingredient, B was the coefficient generated from the model, subscript represented the ingredients.

Areas of overlap, where all responses were simultaneously optimized, were determined. The best formulation was chosen through the desirability function (Baş and Boyaci, 2007; Di Monaco et al., 2010; Miele et al., 2015). Optimal conditions were ascertained by preparing the sample with the highest desirability in the optimized region and by determining significant differences between observed and predicted values (paired t-test, $p \leq 0.05$) (SPSS v. 17.0 for Windows ®).

3.2. Results and discussion

3.2.1 Particle Size Distribution

The PSD curves obtained for three different formulations (17, 4, 13), at low (0.32), medium (0.42), and high (0.51) Oil/Powder ratio, are reported in **Figure 3.1**.

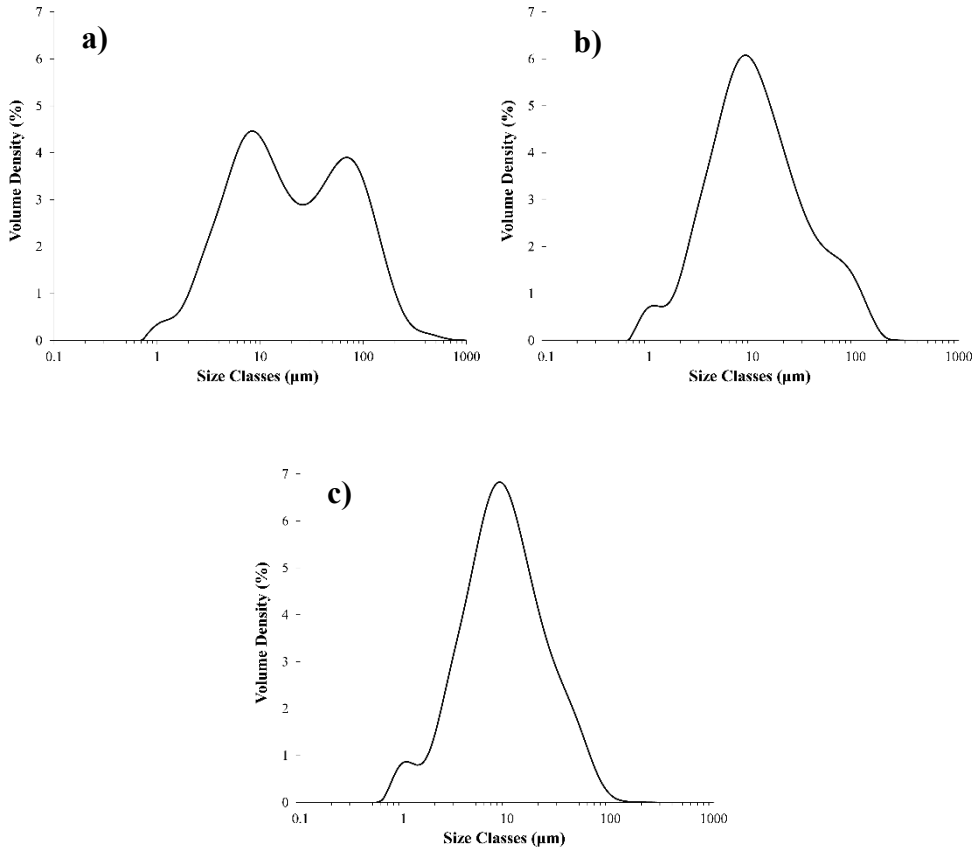

Figure 3.1. Particle Size Distribution of three different formulations
a) Form. 17 (low Oil/Powder ratio); b) Form. 4 (medium Oil/Powder ratio); c) Form. 13 (high Oil/Powder ratio)

As the Oil/Powder ratio increased the PSD changed; in particular, a bimodal distribution was observed (**Figure 3.1.a**), then the second peak of the bimodal became a shoulder (**Figure 3.1.b**) and finally a unimodal distribution was obtained (**Figure 3.1.c**). As stated by Bolenz and Manske (2013), unimodal

PSD are more typical for ball mill refining products, differently from many roller-milled products which present a multimodal PSD. Unimodal distributions were observed for milk chocolate (Bolenz et al., 2014) and for anhydrous paste for ice creams (Fidaleo et al., 2017) refined in ball mills.

In the next page, **Table 3.1.** reports Oil/Powder ratio, Oil/Icing Sugar ratio, the percentiles of the PSD, and rheological parameters for each formulation of the experimental design.

Granulometry is the most closely related feature of the cream structure (Afoakwa et al., 2008; Glicerina et al., 2014). From the parameters reported, the effect of Oil/Powder ratio on the Particle Size Distribution was evident: the lower the Oil/Powder ratio, the coarser the granulometry of the cream. In particular, high D_{90} values were observed for form. 8, 12, 16, 17 (> 90 μm). Conversely, 2, 5, 11, 13, 18, 19 formulations showed significantly lower D_{90} values (between 36 and 46 μm). D_{50} and D_{10} were less affected by the Oil /Powder ratio. Furthermore, there is also an effect of Oil/Icing Sugar ratio on D_{90}, in fact the highest Oil/Icing Sugar ratio the lowest D_{90} value (e.g. comparing Form. 2 and 13). A D_{90} value of about 40 μm is desirable (Afoakwa et al., 2007), considering that for a similar product, anhydrous paste for ice creams, at 42 μm D_{90} value corresponded a very small graininess in the mouth (Fidaleo et al., 2017). Plumpy' Nut ®, actually the most diffused RUTF, showed a D_{90} around 190 μm.

Considering that at small variations of the RUTF formulation, in terms of oil, powder and type of powders, correspond wide variations in terms of Particle Size Distribution, it is crucial to choose the right combination of oil, flour and icing sugar.

Table 3.1. Oil/Powder ratio, Oil/Icing Sugar ratio, mean values of D_{10}, D_{50}, D_{90}, K and n indexes, for each RUTF formulation.

Formulation	Oil/Powder	Oil/Icing Sugar	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	K (Pa s^n)	n
1	0.47	1.01	2.86±0.03^{g}	9.98±0.2018^{cde}	48.16±4.1918^{bc}	57.57±2.3328^{cdef}	0.47±0.0129^{cdefg}
2	0.51	1.00	2.74±0.08^{b}	9.90 ±0.3213^{cde}	42.45±6.1413^{c}	53.69±5.2766^{cde}	0.40±0.0273^{abc}
3	0.48	1.21	3.06±0.03^{l}	10.68±0.1473^{f}	59.27±3.1650^{de}	75.20±4.5529^{g}	0.42±0.0042^{abcd}
4	0.42	0.89	2.90±0.0336^{h}	10.16±0.1830^{de}	52.22±4.9143^{cd}	107.41±2.1337^{i}	0.40±0.0048^{ab}
5	0.51	1.13	2.78±0.0336^{cd}	9.66±0.2120^{bc}	45.30±3.5422^{bc}	60.06±4.5207^{cdef}	0.40±0.0167^{ab}
6	0.45	0.91	2.80±0.0246^{de}	9.96±0.1360^{cde}	53.09±3.7849^{cd}	70.43±2.3203^{fg}	0.42±0.0113^{abcde}
7	0.42	1.05	3.00±0.0639^{i}	10.22±0.1908^{de}	52.70±3.2274^{cd}	66.5±3.1061^{efg}	0.38±0.0729^{a}
8	0.32	0.71	3.73±0.0866^{a}	19.21±2.0395^{l}	128.75±41.1402^{i}	110.76±15.9953^{i}	0.45±0.0708^{abcdefg}
9	0.51	1.19	2.84±0.0487^{fg}	9.81±0.1573^{cd}	47.55±2.9332^{bc}	47.36±4.6889^{abc}	0.44±0.0119^{abcdefg}
10	0.44	1.00	2.93±0.0742^{h}	10.15±0.2826^{de}	52.71±5.3579^{cd}	63.1±6.1226^{defg}	0.44±0.0164^{abcdefg}
11	0.51	1.40	3.09±0.0305^{l}	10.34±0.1353^{ef}	41.83±3.6321^{bc}	39.61±1.6202^{a}	0.46±0.0076^{bcdefg}
12	0.37	0.87	3.67±0.0378^{n}	16.27±0.6976^{i}	95.73±8.5204^{g}	56.66±18.6125^{cdef}	0.49±0.0321^{fg}
13	0.51	1.40	2.76±0.0210^{bc}	9.10±0.1673^{a}	35.78±2.8833^{a}	35.75±2.1067^{ab}	0.47±0.0102^{defg}
14	0.45	0.91	2.92±0.0514^{h}	12.15± 0.3692^{g}	66.39±6.9962^{ef}	60.35±2.7107^{cdef}	0.50±0.0129^{g}
15	0.47	1.11	2.74±0.0309^{b}	9.75±0.3205^{bcd}	48.14±7.6814^{bc}	50.89±3.6621^{bcd}	0.47±0.0106^{cdefg}
16	0.37	0.87	3.64±0.0920^{n}	15.82±1.4398^{h}	92.12±25.6673^{g}	98.65±12.6106^{h}	0.42±0.0687^{abcde}
17	0.32	0.71	3.71±0.0800^{a}	18.87±1.1829^{l}	117.05±12.3266^{h}	112.94±7.7114^{i}	0.48±0.0644^{defg}
18	0.51	1.00	2.82±0.0254^{ef}	9.86±0.1531^{cde}	45.76±3.1505^{bc}	48.65±3.1127^{abc}	0.43±0.0103^{abcdef}
19	0.51	1.06	2.66±0.0226^{a}	9.35 ±0.0955^{ab}	40.24±1.2746^{ab}	38.67±4.1995^{ab}	0.44±0.0177^{abcdefg}
20	0.37	0.80	3.18±0.0322^{m}	11.95±0.2724^{g}	69.33±8.1556^{f}	69.95±3.8909^{fg}	0.49±0.0136^{efg}

Data represent means ± standard deviations of three replicated determinations. Values in the same column followed by different letters differ significantly at $P<0.05$ level (Duncan's method).

3.2.2 Viscosity

All the formulations exhibited pseudoplastic behaviours as shown in **Figure 3.2**. The apparent viscosity decreased from about 100 Pa s to ≈ 10 Pa s as the shear rate increased from 2 to 50 s^{-1} At low shear rate values (2 s^{-1}) a relationship between Oil/Powder ratio and viscosity was found. In particular, looking at the **Figure 3.2.**, at higher Oil/Powder ratio lower viscosity values corresponded. Instead, at higher shear rate values (50 s^{-1}) the Oil/Powder ratio effect was not evident.

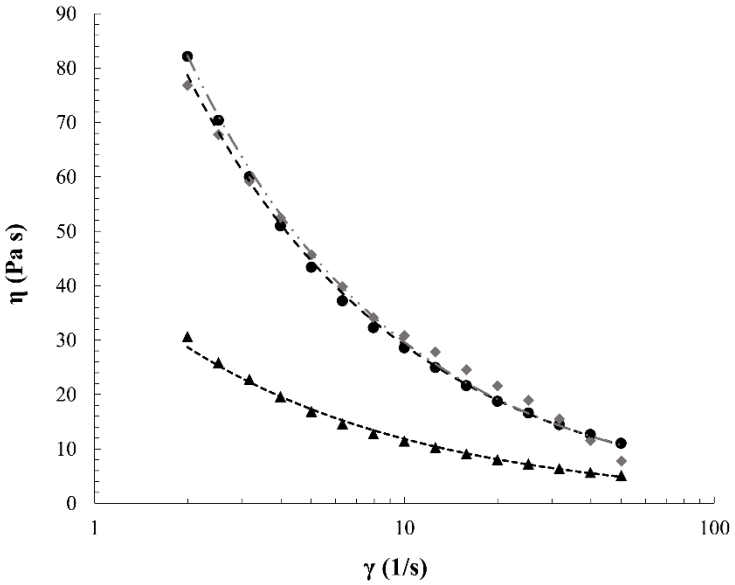

Figure 3.2. Apparent viscosity (η) as a function of shear rate ($\dot{\gamma}$) for formulations 17-4-13

Symbols refer to experimental data while the continuous lines where calculated by using the Power Law model with the parameter estimates reported in Table 1.

◆ Form. 17 ● Form. 4 ▲ Form. 13

All samples were characterized by n values in the range 0.38-0.5 typical of pseudoplastic fluids (**Table 3.1.**). The K values were in the range 35-113 Pa s^n and tended to increase as Oil/Powder ratio decreased. A consistency index lower than 400 Pa s^n is required for cream products to be squeezed (Sun and Gunasekaran, 2009); meanwhile peanut butter products are easily swallowed

if K is lower than 100 Pa s^n (Campanella and Peleg, 1987. Bot et al., 2003). Despite Plumpy' Nut ® RUTF shows a peanut butter consistency, it is considered unacceptable by more than 30% of children (Ali et al., 2013).
Formulations with high amounts of powders and less oil (4, 8, 16, 17) presented high K values, and therefore a consistency too high, unacceptable. Formulations with high oil content (11, 13, 19), on the other hand, had K indices similar to those of medium-oil content creams (1, 2, 9).
All the formulations presented low a_w values, around 0.51±0.04 and there was no effect of the formulation on this parameter. Furthermore, this value is under WHO specifications for RUTF formula (Santini et al., 2013).

3.2.3 Formula optimization

Predicted models were carried out in order to determine a relationship between predictor variables (mix flour, lipid phase and icing sugar content) and the physical properties of cream samples. The only cream sample properties that discriminated the analysed formulations (D_{10}, D_{50}, D_{90}, K) were used as response variables for the mathematical models built to determine the optimal levels of ingredients.
Response variables were explained through linear and cubic models. In particular, cubic models were used to explain PSD parameters; meanwhile a linear model was used to estimate K value. Estimated parameters of the regression models are listed in **Table 3.2.** (in the next page), for each response variable; the results achieved for the regressions in terms of R^2 are also presented in the same **Table 3.2**. The high value of R^2 indicates the good performance and precision of the models for D_{10}, D_{50} and D_{90}; the worst estimation was obtained for K parameter. Linear coefficients (mix flour, lipid phase, and sugar content) affected more D_{90} parameter than D_{50} parameters, in

fact the regression model coefficients, in absolute value, were very high (+62 for flour, -133 for the lipid phase, +175 for sugar) for D_{90}.

Table 3.2. Parameter estimates for variables used in prediction model for PSD and K parameters of RUTF.

Variables	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	K (Pa s^n)
Mix Flour	-1.0027	+17.1946	+62.1540	+2.0330
Lipid phase	+5.1372	-6.3156	-133.0232	-3.2894
Icing Sugar	-1.6771	+6.6689	+175.2783	+3.0471
Mix Flour*Lipid phase	-0.0634	-0.0977	+1.5132	-
Mix Flour*Icing Sugar	-	-	-	-
Lipid phase*Icing Sugar	-	+0.1875	-0.9800	-
Mix Flour*Lipid phase* Icing Sugar	-	-	-	-
Mix Flour*Lipid phase* (Mix Flour-Lipid phase)	-	-0.0182	-0.1338	-
Mix Flour*Icing Sugar* (Mix Flour-Icing Sugar)	-	-	+0.1185	-
Lipid phase*Icing Sugar* (Lipid phase-Icing Sugar)	$-2.6848 \cdot 10^{-3}$	-0.0168	-	-
R^2	0.9406	0.9684	0.9577	0.6430
Significativity	0.0001	0.0001	0.0001	0.0002

For each prediction model only significant parameter estimates are reported ($P < 0.05$).

Predicted models were used to generate contour plots for parameters significantly predicted by independent variables. In **Figure 3.3.**, as an example, the contour map for D_{90} and K index are reported. The contour plots have contour lines that represent constant values of each response variable.

a) **b)**

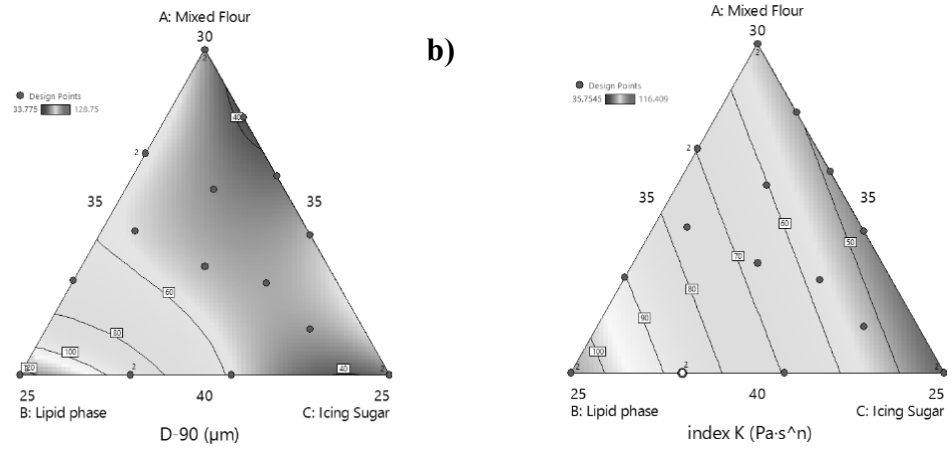

Figure 3.3. Contour maps of some predicted parameters
a) Contour map for D_{90}; b) Contour map for K index
Contour lines on each map represent constant values of each response variable. A contour line, corresponding to an optimal value of each response, may be selected on each map to obtain desirable levels of ingredients

A contour line, corresponding to an optimal value of each response, may be selected on each map to obtain desirable levels of ingredients (Gacula, 1993; Di Monaco et al., 2010). **Figure 3.3.a** shows that D_{90} value increased as the powder concentration (flour and sugar) increased too, whereas it decreased as the lipid phase decreased too (**Figure 3.3.a**). K index increased as the powder increased or decreased proportionally with the presence of oil (**Figure 3.3.b**), determining a different consistency of the different RUTF formulations.

The best formulation of model RUTF was found, through the desirability function, imposing as constraints the minimization of both D_{90} and K index. Four optimized formulations came out and their D_{10}, D_{50}, D_{90}, K index and desirability are reported in **Table 3.3**.

Table 3.3. Solutions for optimum level of ingredients and predicted values.

Predicted formulation	Oil/Powder	Oil/Icing Sugar	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	Index K (Pa s^n)	Desirability
Optimized 1	0.54	1.40	2.94	9.10	38.93	42.38	*0.918*
Optimized 2	0.54	1.03	2.78	9.81	43.55	51.08	*0.810*
Optimized 3	0.54	1.00	2.61	9.97	48.29	52.51	*0.792*
Optimized 4	0.47	1.14	2.84	9.10	45.67	60.59	*0.692*

In italics are indicated the best solutions.

Optimized formulation 1, with the highest desirability (**Table 3.3**), was used to validate the model (Di Monaco et al., 2010; Miele et al., 2015). The results from paired t-test showed no significant differences ($p \leq 0.05$) between predicted and measured parameters for PSD parameters and K index (**Table 3.4.**).

Table 3.4. Response variables measured on each optimal formulation.

Formulation	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	K (Pa·s^n)
Optimized 1	2.73 ± 0.06^{a}	9.08 ± 0.05^{a}	37 ± 1.63^{ab}	38.36 ± 0.54^{a}

Duncan test applied to verify difference among the formulations for each parameter $p \leq 0.05$.

In **Figure 3.4.** the properties of the first optimal formulation are reported. This formulation presented a unimodal PSD curve with low D_{90} values, and a viscosity curve very similar to formulation number 13 of the experimental design, with a high Oil/Powder ratio (**Figure 3.4.** vs. **Figures 3.1.-3.2.**).

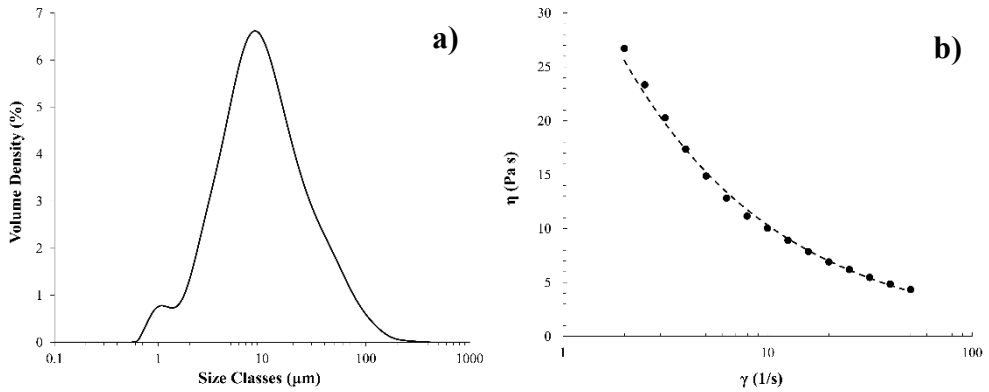

Figure 3.4. Properties of first optimal formulation
a) PSD; b) Apparent viscosity as a function of shear rate

3.3. Conclusions

Starting from world's need to fight the child malnutrition, the present study tried to develop a new RUFT by using ingredients with a low cost and easily to find in sub-Saharan Africa. In order to do that, a formula optimization approach was used, by varying the level of key ingredients in a range ensuring nutritional content expected for the final product. D-Optimal design was useful to study the effect of icing sugar, mix flour and oil content on properties of RUTF models. The strengths of this method lie in reducing the number of formulations to run. Through this method it has been verified that the consistency and the particle size are strongly affected by the mutual relations between the ingredients, in fact, with the desirability function, it was possible to define an optimal RUTF formula. This approach is very interesting because it is fully scalable and adaptable to different types of ingredients. This is

essential for adapting the production of alternative RUTF to the specific social and economic environment of each individual region. Therefore, the optimization of the formula analyzed in this paper turns out to be flexible and potentially efficient from an economic point of view.

On the other hand, however, further studies will have to be carried out for the implementation in the formula of a possible mix of mineral salts, to reach the micronutritional specifications recommended for this type of product. Finally, once the level of sustainable scales for rural realities is defined, it will be necessary to evaluate the clinical efficacy and acceptability of the hypothesised optimal RUTF.

3.4. References

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4. Lipid Oxidation Analysis on the alternative optimized RUTF formulations at a small scale set

A first part of this chapter is dedicated to the validation of the prediction model proposed on the batch used, specifically, for lipid oxidation experiment. It was important to verify the adherence of the model with the values measured on the response variables, in order to be sure that the output informations would have been correct. With the aim to define the oxidation stability of the alternative RUTF formulations, an experimental design based on the analysis of *primary* and *secondary oxidations* was carried out on all four optimized formulations and explained as follows.

4.1. Materials and Methods

As written in the previous chapter, after the D-Optimal Response Surface variable procedure (considering Particle Size Distribution D_{10}, D_{50}, D_{10}, a_w, K and n viscosity parameters), four optimal Ready-to-Use Therapeutic Food (RUTF) formulations were predicted (**Table 4.1.**).

Table 4.1. Optimal formulations individuated by the D-optimal Response Surface experimental design at three factors.

Predicted formulation	Oil/Powder	Oil/Icing Sugar	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	Index K (Pa s^n)
Optimized 1	0.54	1.40	2.94	9.10	38.93	42.38
Optimized 2	0.54	1.03	2.78	9.81	43.55	51.08
Optimized 3	0.54	1.00	2.61	9.97	48.29	52.51
Optimized 4	0.47	1.14	2.84	9.10	45.67	60.59

Predicted formulations were produced again, to evaluate the response variables of the previous experiment and the lipid oxidation. In particular, Thiobarbituric Acid Reactive Substances (TBARS) [with the production of Malondialdehyde (MDA)] and conjugated dienes analyses were conducted as an oven

accelerated test at 30, 40 and 60 °C, in presence of infinite oxygen, to simulate a brute production. The same ingredients of the previous chapter were used, adopting equivalent treatments and methods (**§ 3.1.1.**). Three replications for each sample were prepared.

4.1.1. Recalls of the previous chapter

Spread refining was performed using a PM-200 (Retsch, Germany) ball miller with five 2 mm balls and four 1 mm balls, for 120 minutes at 500 rpm (Armini, 2016). Particle Size Distribution (PSD) was carried out using a Mastersizer 3000 (Malvern Instruments, UK), with sunflower oil as dispersant (1.469 R.I.), 1.51 Particle Refractive Index, 0.1 Particle Absorption Index, Obscuration 18-20%, no sonication (Afoakwa et al., 2008). The flow curves were obtained using ARES-LS rheometer (Rheometrics Inc., USA), working at 30 °C, at a shear rate of 0.1 s^{-1} - 100 s^{-1}, pre-shear of 30 s. Water activity was evaluated by an Aqualab-Dew point water activity meter (4TE, USA). The instrument was calibrated between 0.5 and 0.76 values, at a temperature of 20 °C.

4.1.2. Methods

4.1.2.1. Colour

Colour was evaluated by a CR-300 colorimeter (81981058, Japan). An initial calibration on the white and, subsequently, 2 measures on 3 different aliquots of each formulation, evaluating the coordinates L *, a *, b *, which define:
L * = brightness; a * = - to green; + to red; b * = - blue b; + b yellow

4.1.2.2. Preparation of MDA standard

TBARS analysis was carried out according to Maraschiello et al. (1999).
73.2 mg of Tetraethoxypropane (TEP) were taken for the preparation of the MDA standard to which 10 mL of 0.1 N HCl were added. The tube was immersed in a bath for 5 minutes at 100 °C and subsequently cooled. The content of this was increased to 100 mL, resulting in a solution with an MDA concentration of 239 µg / mL. For the definition of the calibration line a 100 µg / mL solution is required; therefore, by applying Boyle's law, whereby c · v = C1 · V1, there were 239 mL of MDA diluted at a concentration of 100 µg / mL (Botsoglou et al., 1994). In this case, the calibration line was constructed with dilutions 1: 2 (2 mL of the 100 µg / mL solution of MDA and 2 ml of bidistilled water), 1: 5 (800 µL of the solution at 100 µg / mL of MDA and 3.2 mL of bidistilled water), 1:10 (400 µL of the solution at 100 µg / mL of MDA and 3.6 mL of bidistilled water) and 1:20 (200 µL of the solution at 100 µg / mL of MDA and 3.8 mL of bidistilled water).
1 mL of the prepared solutions was added to 10 mL of bidistilled water and vortexed for 30 seconds. 2.5 mL of 25% Trichloroacetic Acid (TCA) were added, then the tubes were mixed and stored at 4 ° C for 30 minutes. Subsequently, the solutions were centrifuged for 5 minutes at 4000 rpm at 4 ° C. 3.5 mL of supernatant were recovered and 1.5 mL of 0.6 % Thiobarbituric Acid (TBA) were added. Finally, they were stored in a bath at 70 °C for 30 minutes. These preparations were diluted 1:5 to respect the linearity of the absorbance reading. The samples were read with Spectrophotometer (Shimadzu UV-1601, Japan) at 532 nm against a white consisting of 10 ml of bidistilled water, 2.5 ml of 25% TCA and 1.5 ml of 0.6% TBA.

4.1.2.3. TBARS Test

1 g of sample was taken, placed in tube, and added to 10 mL of bidistilled water and 5 mL of hexane. The mixture was treated with Ultraturrax for 1 minute. To this were added 2.5 mL of 25% TCA and the solution, after being vortexed for 1 minute, was stored for 30 minutes at 4 °C. Subsequently, the samples were subjected to centrifuge (THERMO Scientific IEC CL30R Centrifuge, Italy) at 4000 rpm for 5 minutes. After removing the excess with hexane, an aliquot of 3.5 mL of supernatant was added to 1.5 mL of 0.6% Thiobarbituric acid and incubated in a bath for 30 minutes at 70 ° C. After being cooled, the samples were diluted 1:5 to comply with the linearity parameters of the calibration line. The intensity of color development was measured with a spectrophotometer at 532 nm, against a white consisting of 10 mL of bidistilled water, 2.5 mL of 25% TCA, 5 mL of hexane and 1.5 mL of 0.6% TBA, all treated according to the same conditions as the sample.

4.1.2.4. Conjugated dienes

1 gram of each replication was mixed with 20 mL of a 2:1 chloroform / methanol solution, after which the samples were stirred for 20 minutes on the tilting plane and centrifuged for 10 minutes at 4000 rpm. The supernatant was recovered, while the remaining pellet was subjected twice to the same steps described above. The total supernatant was treated with an aqueous solution of 0.5% sodium chloride, in order to separate the lipid phase from the hydrophilic phase by means of a separating flask. Following a further and appropriate dehydration with sodium sulphate, the lipid phase was inserted into a flask (of which the empty weight was recorded) and dried with a rotating vacuum evaporator. This was a modified lipid cold extraction method proposed by Bligh and Dyer, 1959. Then, 0.1 g of the lipid fraction was inserted into a 10

mL flask and brought to volume with hexane, obtaining a known concentration (1%). After taking 1 mL of this solution, it was placed in another 10 mL flask to obtain a 0.1% solution, always bringing to volume with hexane. Subsequently it was read at the spectrophotometer at a wavelength of 232 nm. Data were compared and evaluated by a Duncan test with $p \leq 0.05$ significance, using SPSS v.17.0 on Windows 7 operating system.

4.2. Results and Discussion

In **Tables 4.1.** and **4.2.** (in the next page), technological response variables, measured on four optimal RUTF formulations, are shown. In particular, significant PSD D_{10} e D_{50} variations weren't found; D_{90}, instead, was a significant variable among RUTF formulas. D_{90} is a parameter strongly correlated to the rheological behaviour and acceptability of a spread and its value should be under a value of 60 µm and, better, under a value of 35 µm (Afoakwa., 2007). From this point of view, all spreads were acceptable, while the best spread was the "4 opt". Plumpy' Nut ® was very far from this value, with a D_{90} of 178.5 µm.

K parameter is very important to define the deglutition attitude of a spread. In any case, $K < 100$ Pa·s^n is considered optimal (Bot et al., 2003). K values of all optimal formulations were under this value, so they were considered suitable for feeding purpose.

Water activity (a_w) was under the safe value of 0.6 (Santini et al., 2013) in all optimal formulations and also in Plumpy' Nut ®.

Finally, regarding color parameters (L, a and b), a value was strongly correlated to the quantity of sugar in the recipe.

Table 4.1. Response variables measured on each optimal formulation ().

Formulations	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	K (Pa·s^n)
1 opt	2.83±0.01^a	9.06±0.04^a	36±1.15^{ab}	39.24±0.42^a
2 opt	2.65±0.02^{bc}	9.42±0.10^a	41.8±1.87^b	38.50±4.11^c
3 opt	2.62±0.02^b	9.27±0.10^a	38.1±1.63^{ab}	42.13±2.22^c
4 opt	2.715±0.01^c	8.75±0.05^a	33.4±0.705^a	30.25±1.129^b
Plumpy' Nut	3.78±0.12^d	46.55±5.08^b	178.5±16.6^c	/

Duncan test applied to verify difference among the formulations for each parameter $p \leq 0.05$.

Table 4.2. Response variables measured on each optimal formulation (Duncan test applied to verify difference among the formulations for each parameter $p \leq 0.05$).

Formulations	a_w	L	a	b
1 opt	0.50 ± 0.02^a	22.74 ±0.14^a	-2.59±0.14^a	10.93 ± 0.11^a
2 opt	0.59 ± 0.01^b	24.42 ±0.28^d	-3.19±0.15^b	13.29±0.33^d
3 opt	0.54 ± 0.01^{ab}	24.27±0.07^c	-3.47±0.05^c	12.77±0.08^c
4 opt	0.52 ± 0.06^a	23.53±0.08^b	-3.40±0.42^c	12.41±0.56^b
Plumpy' Nut	0.52 ± 0.01^a	69.80±0.297^e	2.57±0.0781^d	27.98±0.33^e

Duncan test applied to verify difference among the formulations for each parameter $p \leq 0.05$.

In order to evaluate the oxidation stability of the four RUTF formulations, an oven test at 30, 40 and 60 °C was carried out. As shown in **Figure 4.1.a** and **Figure 4.1.b** (in the next page), at 30 °C and 40 °C, peroxydienes grew up slowly in 2 – 3 opt and 1 – 4 opt couples of formulations, showing a similar behavior, respectively. At 60 °C, after a first week of induction, a greater rate of primary oxidation in all optimal formulations was observed (**Figure 4.1.c –** in the next page). In general, ***2 and 3 opt formulations were more stable than the others under the perspective of primary oxidation***.

Regarding secondary oxidation (MDA), **Figures 4.1.d, 4.1.e** and 4.**1.f** (in the next page) showed a substantial statistical similarity among all four optimal formulations. In particular, at 30 °C and 40 °C MDA formation started in sensible way only after the second week. In general, MDA formation in our

analysis temperatures was very slow. This result can be extremely important in perspective to set a local RUTF production and distribution at room temperature, without a complicate packaging system.

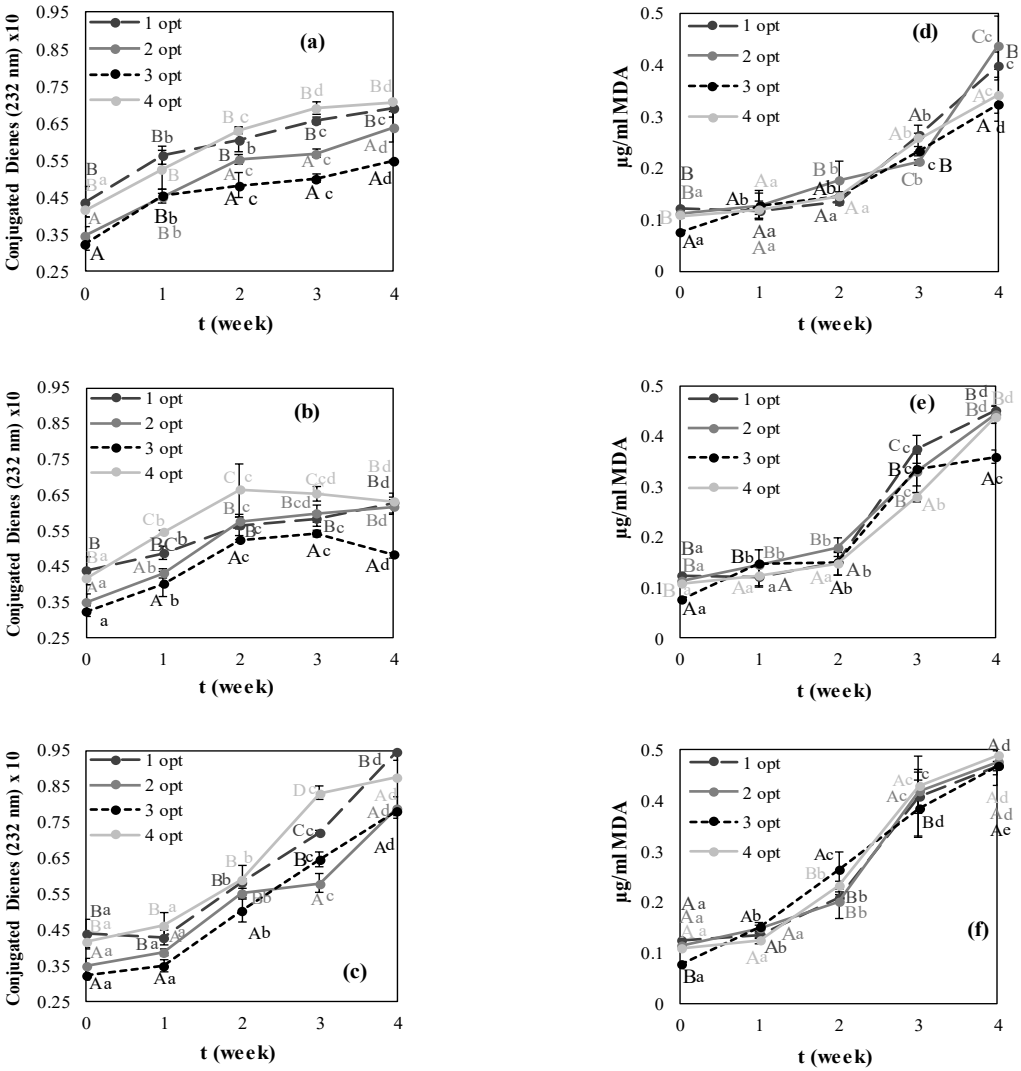

Figure 4.1. Accelerated oxidation test (30, 40 and 60 °C) on four optimal RUTF formulations. **a)** Primary oxidation (peroxydienes) at 30 °C for 4 weeks; **b)** Primary oxidation (peroxydienes) at 40 °C for 4 weeks; **c)** Primary oxidation (peroxydienes) at 60 °C for 4 weeks; **d)** Secondary oxidation (MDA) at 30 °C for 4 weeks; **e)** Secondary oxidation (MDA) at 40 °C for 4 weeks; **f)** Secondary oxidation (MDA) at 60 °C for 4 weeks. Small letters show the differences during the time; capital letters show differences among samples at the same time; Duncan test ($p \leq 0.05$).

4.3. References

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5. Non-oral and oral sensory evaluation of alternative RUTF

A concern of the already developed RUTFs is the acceptability, especially that of peanut-based RUTF for the treatment of CMAM (Nga et al., 2013). In literature, several RUTF acceptability trials are reported, some of them included moderate acute malnutrition children, other healthy children and others adult people. An acceptability trial in Cambodia with Plumpy' Nut ®, showed that it was not well accepted by Cambodian children (Bourdier, 2009*).* Multiple acceptability trials in South Asia have shown limited acceptability of RUTF and demonstrate a need for more culturally accepted ingredients and formulations (Ali et al., 2013; Nga et al., 2013). A novel milk-free soybean–maize–sorghum RUTF was liked by healthy children in Zambia (Owino et al., 2014), so then a clinical trial was performed (Irena et al., 2015) to compaire this new RUTF with peanut based RUTF. The use of country-specific formulations has the potential to increase the practice of community-based treatments, therefore reducing the morbidity and mortality of SAM in settings where RUTF is not widely used (Weber et al., 2017; Brixi, 2018).

The first objective of the present part was to produce these 4 optimal formulations in a pilot refining machine and to characterize them from different points of view. In total 4 RUTF creams were prepared and rheological properties, particle size distribution, microbiological determination, volatile compounds, oxidation indexes and sensory properties were determined in order to predict and/or select the best formulation. Finally, because the final objective of the work was to obtain a product ensuring nutritional content expected for the final product, but also liked and easily accepted by both the children and their parents, a preliminary acceptability test was performed, by involving only adult subjects (instead of children too) as first step of the investigation.

5.1. Materials and Methods

5.1.1. Sample preparation

The adopted ingredients, as we reported in the paragraph 1.2, were dehulled soy, dehulled sorghum, sugar, sunflower oil, soy lecithin and Arthrospira maxima (Spirulina) dried powder. The treatments, carried out on raw materials, were defined in order to obtain powders suitable for the cream production process. For soy and sorghum, it was necessary to roast, to reduce the water activity and to degrade the anti-nutritional components. For these reasons, the thermal history defined was 120°C for 3 hours in a fluid bed dryer. Subsequently, the grinding step was optimized for both the roasted seeds and the dried Spirulina in a PM-200 Ball Miller (Retsch, Germany). Raw roasted seeds were initially grinded at 1 mm cut off, then at 250 µm; Spirulina directly at 250 µm. The purpose of scaled up production was to emulate local technological conditions, without availability of transformed ingredients such as flours. For cream production, a stirred ball mill was used (model Micron 20, Selmi s.r.l, Modena, Italy). 60 kg spheres of 5.5 mm diameter were used. Ingredient weight was set on 10 kg, according to manual specifications of the instrument. Speed rate was fixed (72 rpm) and the temperature was controlled (32 ± 3°C). Refining time was 240 min for all the formulations.

5.1.2. Technological characterization

Particle size measurements, viscosity and a_w of 4 samples were performed as already described in *3.1.1.1*, *3.1.1.2*, *3.1.1.3* subparagraphs.

5.1.3. Qualitative assessment of volatile aroma components

Those determinations were performed on 4 optimized RUTF (F1, F2, F3, F4) and also on Plumpy' Nut ® (PL).

5.1.3.1. Dynamic headspace-solid phase microextraction (SPME) and gas chromatography/mass spectrometry analysis (GC/MS)

The volatile compounds were extracted and concentrated by SPME and analyzed by a GC/MS system (Genovese et al., 2015). Extraction and concentration of volatiles was carried out with a divinyl-benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (50/30 µm thickness and 1 cm length stationary phase) (Supelco, Bellefonte, PA). RUTF samples (1 g) were equilibrated for 30 minutes in a 15 mL pierceable vial at 40 °C and for 10 minutes in the presence of the SPME fiber in vial headspace (Vichi et al., 2003). After the equilibration time, the fiber was placed in the injection port of the GC/MS system where it was desorbed in splittless mode for 10 minutes at 230 °C. Volatile compounds were analyzed by GC/MS using a QP5050A (Shimadzu, Kyoto, Japan) equipped with a Supelcowax-10 capillary column (60 m, 0.32 mm i.e., 0.5 µm thickness) (Supelco). Temperature was set at 40 °C for 4 min, followed by an increase of 3.5 °C/min up to 240 °C, and held for 3 min at maximum temperature. The injector was kept at 250 °C. Helium was used as a carrier gas (1.4 mL/min). Volatile compounds thermal desorption was carried out by exposing the SPME fiber in the injector for 10 min. The compounds identification was performed by comparing retention times and mass spectra obtained by analyzing pure reference compounds in the same conditions. Moreover, the identification was confirmed by comparing mass spectra with those of the NIST database. Mass

spectra were recorded at 70 eV. Source temperature was 200 °C and the interface temperature was 250 °C

5.1.3.2. Head space analysis by Electronic Nose.

To analyze RUTF volatiles in the static headspace, an Electronic Nose (Airsense Analytics, Germany) PEN2 with 10 MOS (Metal Oxide Semiconductor) sensors was also used (Battimo et al., 2007). The operating conditions were: the baseline was acquired in a filtered dry air in a continuous flow of 600 mL/min for 300 seconds; aliquots of 1 g of each RUTF samples have been introduced in 20 mL vials with a pierceable Silicon/Teflon cap and incubated at 30 °C for 30 min before injection. After a headspace generation, the volatile compounds have been directly transferred by the carrier gas, at a constant flow rate (400 mL/min), into the sensors chamber and the sensor responses acquired for 100 sec. Then, sensors have been exposed to air in order to keep the gas sensor signal back to the baseline. Sensor responses towards RUTF samples were analyzed by the software Winmuster vers.1.6 (Airsense Analytics, Germany).

5.1.4. Oxidation indexes

Those indexes were measured as already described in subparagraphs *4.1.2.2*, *4.1.2.3*, *4.1.2.4*. Primary and secondary oxidation indexes were measured only at one storage time (t_0) and only at one temperature (30 °C).

5.1.5. Sensory analysis

5.1.5.1. Non oral sensory evaluation

Panelists. 9 healthy selected panelists (7 females and 2 males, 20–27 years old, mean age 23.1 ± 1.9) were recruited for sensory evaluation. All panelists have healthy dentition and normal occlusion. They were required to undergo two training sessions. Judges were trained for approximately 10 h. The first training session was used for attribute generation and evaluation technique definition. The second training session was used to familiarize the panelists with scale and evaluation technique. During the next sessions, the panelists were asked to evaluate the samples in a randomized order. Written informed consents were obtained from all panelists.

Procedure. Visual and tactile texture attributes were evaluated by the panel. Approximately 10 g of each sample was served in two different containers (plastic petri dishes and cups), identified by three-digit random codes and presented in a monadic sequential order. The assessors were provided with a knife and with a spoon for each sample. 9 attributes were scored on 10 cm unstructured lines with references. Attribute definition, evaluation techniques and references are described in **Table 5.1**. (in the next page) and most of them have been kept form Fidaleo et al., (2017) and Di Monaco et al., (2008). Tests were run in separate booths at 20 °C. Assessors evaluated the samples when they reached the temperature of 20 ± 1 °C. Descriptive data were collected over three sessions; each sample was tested following a randomized design with three replications. Data were collected by means of "Fizz Acquisition" software (Biosystemes, Couternon, France).

Table 5.1. Texture and visual profile analysis: attribute definition, evaluation techniques, scale and references.

Attribute	Definition	Evaluation technique	Scale and references
Oily separation	Streaks due to the oil separation	Look at the sample and evaluate the amount of the streaks on the sample surface	No streaks at all (0) (commercial topping) - a lot of streaks (10) (unrefined cream)
Green intensity	Intensity of the green color of the sample	Look at the sample and evaluate the intensity of the green color	Yellow green (0) (unrefined cream) – bottle green(10); (extremely refined cream)
Fluidness	Force required to mix the sample	Dip the spoon in the cup and evaluate the force required to mix the sample	Not very fluid(0) (frozen cream);-intense (8) unrefined; cream; very fluid (10) (*fruit juice)*
Adhesiveness to the spoon	Property of the sample to stick to a surface	Dip the spoon in the cup and then turn of 90° the spoon and evaluate the amount of sample attached to the spoon	Not adhesive at all (0) (water); a little bit adhesive (2) unrefined cream; very adhesive (10) butter and sugar mix
Spreadability	Property of the sample to be spread over a flat surface	Evaluate the ease to spread the sample. Use the rask and the knife to perform the test	Very Difficult/not spreadable at all (0); diffucult (1-2) (butter from the fridge); easy (8) (unrefined cream); very easy (10) mayonnaise
Meltability	Speed of fat melting	Put a spoon of sample in the hand/between fingers and evaluate its melting rate	Not meltable at all (0) (extra dark chocolate); very slowly meltable (2) (dark chocolate); slowly meltable (4) (milk chocolate); medium meltable (6) (refined cream); quickly meltable (10) (butter at room temperature)
Amount of granules	Presence of crystals between fingers	Evaluate the amount of grains between fingers	No grains (0) (commercial topping)-many grains(10) (unrefined cream)
Dimension of granules	Medium size of granules	Evaluate the size of grains between fingers	Very small (0) (icing sugar); small (sugar); medium (brown sugar); very big (10) (coarse salt)
Oiliness	Oily sensation between fingers	After sample melting, evaluate the amount of oil coating the hand	Not oily at all (0) (extra dark chocolate); little oily (5) (milk chocolate); very oily (10) (butter)

5.1.5.2 Preliminary consumer tests

Consumers. A total of 96 subjects (females: 62%; mean age: 35years old) were recruited in Portici (Italy) by means of announcements published on blogs, social networks, emails, pamphlet distribution and word of mouth.

Experimental procedure. At the time of recruitment, respondents were given general information about the study aims and individual written informed consent was obtained from participants. In the days preceding the sensory lab session, respondents were asked to complete at home an online questionnaire aimed at collecting data about socio-demographic. Respondents were asked to declare their own gender, age, presence of food intolerances or allergies (Yes, No), adoption of the own diet for medical reasons (Yes, No) educational level (Lower secondary school; Upper secondary school; Degree; Post-degree),
Tests were conducted individually and social interaction between participants was not allowed.

RUTF acceptability. Subjects were presented with 4 samples coded with a three-digit code. Based on the results of the non-oral sensory evaluation, respondents were asked to rate the intensity of 6 sensory attributes and overall liking for each of the samples by using the General Labeled Magnitude (GLM) scale (Bartoshuk et al., 2002) and Labeled Affective Magnitude (LAM) Scale (Schutz and Cardello, 2001), respectively. In each session, the samples were served at room temperature and presented simultaneously in plastic cups coded with 3-digit numbers. Each sample consisted of 15 g of RUTF cream. The respondents were instructed to eat the entire amount provided prior to rating attribute intensity and liking. An interval of 90 s was imposed between tastings, during which water (mineral water) and a salty cracker was provided for palate cleansing. The sample presentation order was systematically varied according to a William's Latin square.

5.1.6. Data Analysis

PSD and viscosity parameters were calculated as reported by Armini et al., (2018) and Fidaleo et al., (2017). One-way analysis of variance (ANOVA) and multiple comparisons (Duncan) were used to evaluate if formulations differed for those parameters ($p \leq 0.05$) and for non-oral sensory evaluation. The statistical software SPSS for Windows v. 17.0 was used for data analysis (SPSS Inc., Chicago, IL, USA).

PCA e PLS were used to evaluate differences among the samples for their volatile aroma components and oxidation indexes, by using XLSTAT Version 6.1 (Addinsoft, New York, N.Y., U.S.A.).

PLS was also used for consumer test results. The overall liking (dependent variable) (Y) and the perceived intensity of the evaluated sensory attributes (independent variables) (X), for the four formulations (observations) were related using partial least squares regression (PLS).

5.2. Results and discussion

5.2.1. Technological characterization

All the formulations exhibited pseudoplastic behaviours as shown in **Figure 5.1.** The apparent viscosity decreased from about 70 Pa·s^n to ≈ 8 Pa·s^n and from about 30 Pa·s^n to ≈ 3 Pa·s^n as the shear rate increased from 2 to 50/s for formulations 2, 3 and 4 and for formulation 1, respectively.

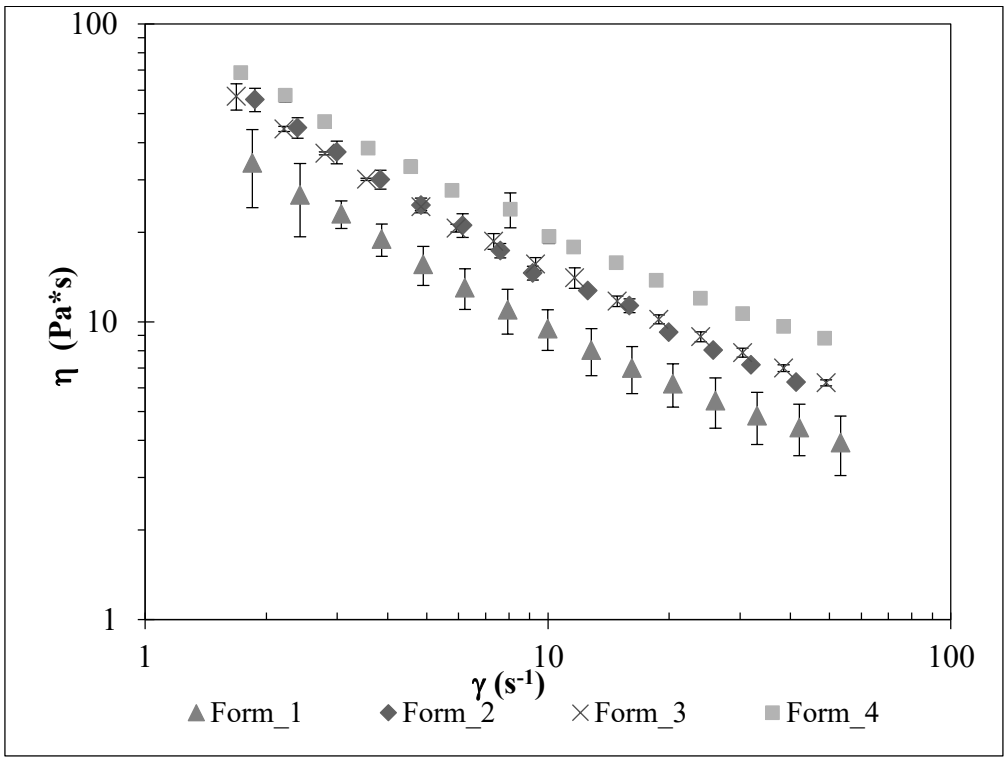

Figure 5.1. Apparent viscosity (η) as a function of shear rate ($\dot{\gamma}$)

All samples were characterized by n values in the range 0.31-0.35 typical of pseudoplastic fluids (**Table 5.2.** in the next page), without significant differences among each other's.

Table 5.2. Mean values (± S.E.) of D_{10}, D_{50}, D_{90}, K and n indexes for each RUTF formulation.

F	Viscosity			Particle size distribution (PSD)		
	K	n	R^2	D_{10}	D_{50}	D_{90}
1	46.93±4.25a	0.337±0.010a	0.987	2.18±0.11	7.58±0.20	25.85±0.37a
2	72.903±4.67b	0.314±0.018a	0.991	2.18±0.11	7.67±0.04	27.75±0.09b
3	70.727±0.74b	0.351±0.008ab	0.993	1.97±0.07	7.29±0.26	26.90±0.58ab
4	87.61±0.61c	0.352±0.002c	0.991	2.16±0.16	7.98±0.06	28.10±0.40b

The K values were around 70 $Pa \cdot s^n$ for the 2 and 3 formulations, with a significant decrease in consistency for formulation 1. Formulation 4 presented the higher k index. Flow index seemed to not distinguish very well formulations, varying from 0.31 to 0.35.

For what concern the PSD parameters, as reported in **Table 5.2.**, D_{90} values varied from 25 to 28 μm.

A consistency index lower than 400 $Pa \cdot s^n$ is required for cream products to be squeezed (Sun and Gunasekaran, 2009); meanwhile products are easily swallowed if K is lower than 100 $Pa \cdot s^n$ (Campanella and Peleg, 1987; Bot et al., 2003) and all the formulations presented a K value lower than 100 $Pa \cdot s^n$; formulation 1 differed from others for consistency due to the different oil/sugar ratio (lower icing sugar content that act as bulking agent) that affect this parameter, meanwhile formulation 4 presented the same soy content of formulation 1 but a lower oil content.

PSD and K values of the RUTF creams prepared in a pilot refining device were not exactly the same of those predicted from our previous work (Armini et al., 2018), but the differences among the formulations were almost the same. It is expected that moving from a small to pilot scale reefing device, the obtained products are not exactly the same ones. However, the obtained results confirmed the efficacy of the scale up process, in fact the consistency index

was lower than 100 $Pa.s^n$ and the D_{90} value was really low for all the formulations.

The efficacy of the scale up process is also underlined by the PSD results, in fact the D_{90} values were lower than 30 µm, critical value for granule size in a cream, because under this value the graininess should not be perceived in mouth.

5.2.2. Qualitative assessment of volatile aroma components

In **Table 5.3.** (in the next page) are reported the main volatile compounds of formulations and reference (PL) with the relative odor descriptors and areas % of the different components identified. The detected volatiles showed a greater presence of molecules deriving from lipid oxidation (Hexanal, Nonanal, Pentanoic Acid, Hexanoic acid, Heptanoic Acid) in the formulations, while the PL profile appeared different, due to the different raw materials used in the production process. The degree of oxidation was more pronounced in formulation 1 which, due to the stress of refining, has released larger quantities of the volatile compounds responsible for oily and fatty off-flavors, deriving from oxidation reactions (Hexanal, Octanal, 1-Octanol, 1-Pentanol). In the planned alternative formulations, the 2,5-Dimethylpyrazine was present, deriving from the roasting of the soy used, with the smell of cocoa, roasted nut, woody, and the 3-(2H)-Furanone, 5-Ethyldihydro, with the smell of herbaceous, tobacco -like, creamy. In the PL, the amine components derived from the degradation of the amino base of proteins, producing protein-based Melanoidins, deriving from non-enzymatic browning reactions (Maillard reaction). The remaining part was characterized by highly aromatic aldehyde fractions, including the Pentenal (green wood); and from very intense alcohol fractions: 1-Hexanol (fruit, floral). In PL there was Dimethylsulfone, which is

an ingredient used as a dietary supplement in substitutive meals, α-Pinene and 1-H-Pyrrole,1-Methyl, with a woody and herbal odor.

Table 5.3. GC-MS/(SPME) results: volatile compounds, peak number and odor descriptors

Odor descriptor	Compound	F1 (area %)	F2 (area %)	F3 (area %)	F4 (area %)	PL® (area %)
Honey	Octanal	-	-	-	-	0.96
	Methyl alcohol	0.08	2.44	1.70	0.42	6.70
	Heptane,2,2,4,6,6-pentamethyl	0.29	3.87	4.00	2.12	3.09
Green	Pentanal	0.15	1.48	2.56	1.36	0.26
	3_Heptene	-	-	-	16.42	3.05
Woody	α-Pinene	-	-	-	-	9.67
Fatty	Hexanal	39.14	17.20	28.12	28.58	13.74
Woody	1-H-Pyrrole,1-methyl	-	-	-	-	6.82
Banana	2-Heptanone	1.54	11.71	-	15.27	-
Oily, Fatty	Heptanal	2.40	2.59	3.00	1.83	-
Green	Furan,2-pentyl	1.32	1.56	1.42	1.38	2.91
Pungent	1-Pentanol	4.04	1.98	2.32	2.43	5.83
Floral	Octanal	5.52	3.17	3.69	3.68	11.35
Nutty	Pyrazine,2,5-dimethyl	0.14	1.28	1.58	3.97	-
Fruity	1-Hexanol	1.27	1.99	6.82	1.85	8.83
Fatty	Nonanal	5.00	6.62	3.94	2.77	-
Berry	3-Octen-2-one	4.52	3.82	8.94	2.12	3.53
	1,3-Hexadiene,3-ethyl-2-methyl	2.28	4.51	3.21	1.96	5.14
Cheese	1-Octen-3-ol	1.65	7.50	2.30	0.40	1.39
Fatty	1-Heptanol	1.26	2.69	0.42	0.62	-
	Decanal	1.77	1.17	0.83	0.09	1.99
Citrus	1-Octanol	1.44	0.69	1.64	1.60	0.43
	Tetradecane,4,11-dimethyl	0.80	0.47	2.56	1.85	1.09
	2-Pyrrolidinone,1-methyl	0.80	1.64	3.57	3.04	-
Fruit	3(2H)-Furanone,5-ethyldihydro	1.64	2.69	1.42	-	-
	Pentanoic acid	0.39	1.97	0.31	-	-
Cheese	Hexanoic acid	0.46	1.16	0.20	-	-
Cooked Milk	Dimethylsulfone	-	-	-	-	13.21
	Heptanoic acid	7.92	1.94	1.34	-	-

Figure 5.2. shows the PLS of the 5 samples, which shows the correlations between volatile substances (Y) and responses of the electronic nose (X).

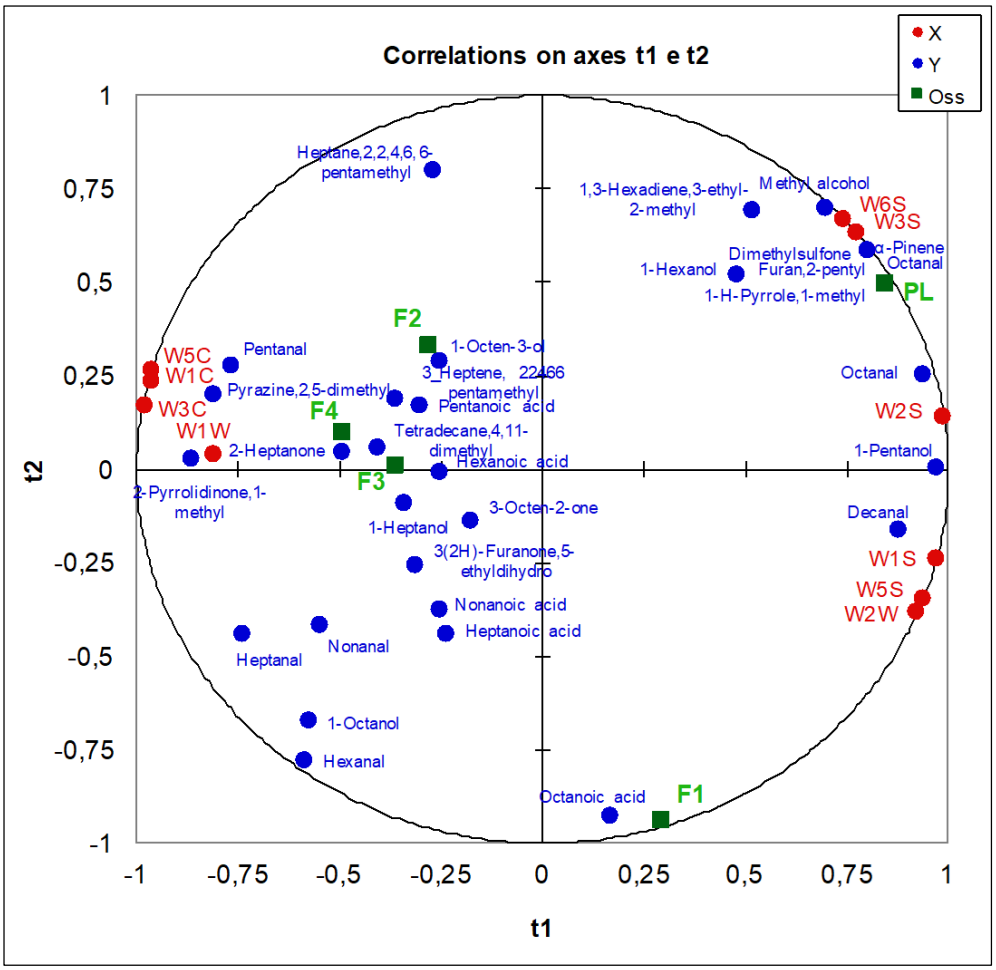

Figure 5.2. NE & GC-MS/(SPME)

PLS Data observations
Data matrix (X): sensors electronic nose
Data matrix (Y): volatile compounds
Dipendent vectors: samples 1-4 and Plumpy'Nut®

Samples were divided in three groups. Formulation 1 was more related to compounds of the degradation of fatty acids, resulting the most oxidized sample. Formulations 2, 3 and 4 were similar to each other and characterized by several classes of compounds (Proteins, Alcohols and Aldehydes). Finally,

PL up was more related to compounds of the degradation of proteins, and not of fatty acids, probably because it was not a refined product.

The correlations between the two axes, t_1 and t_2, made it possible to discriminate the formulations on the basis of both the NE sensors and the content of identified volatile substances. Along the t_1 axis, it is shown that the first formulation was much more oxidized, given the greater presence of compounds deriving from the degradation of unsaturated fatty acid chains, such as: Hexanal, Octanal, Nonanal, 1-Octanol, 1-Pentanol, Nonanoic Acid.

5.2.3. Oxidation indexes

In order to evaluate the oxidation stability of the four alternative RUTF formulations and Plumpy' Nut ®, a test at 30°C was carried out. As shown in **Table 5.4.**, primary (Conjugate Dienes) and secondary oxidation (MDA commonly called TBARS test), at 30 °C were measured.

Table 5.4 Primary and secondary oxidation indexes of samples at 30 °C at t_0.

Samples	Conjugated dienes (Abs @ 232 nm)	MDA (µg/mL)
F1	0.392 ± 0.023^{a}	0.049 ± 0.002^{a}
F2	0.434 ± 0.031^{b}	0.044 ± 0.009^{b}
F3	0.388 ± 0.024^{a}	0.044 ± 0.007^{b}
F4	0.413 ± 0.039^{b}	0.049 ± 0.005^{a}
PL	0.237 ± 0.037^{c}	0.069 ± 0.008^{c}

5.2.4. Sensory evaluation

5.2.4.1 Non-oral sensory evaluation

In the present study only some texture properties of RUTFs were evaluated by assessors. A one-way analysis of variance showed that formulation did not affected some texture attributes, such as oily separation ($p = 0.732$), green

intensity (p = 0.507), spreadability (0.056), granule dimension (p = 0.114) and oily coating (p=0.804). Looking at the **Figure 5.3.**, where those average parameters (± S.E.) are reported, all the RUTFs presented a medium oil separation, were emerald green, easy to spread, with small perceptible granules and oily.

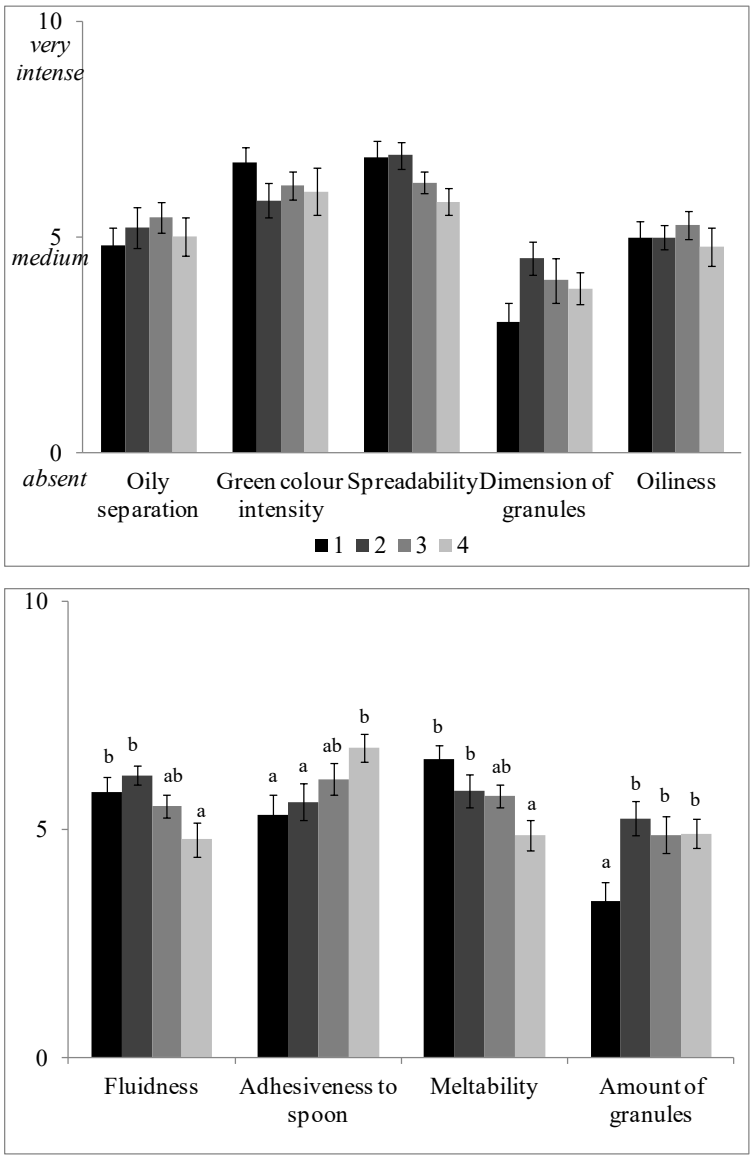

Figure 5.3. Mean values (± S.E.) of non-oral sensory attributes.
a) attributes not affected by formulation (p > 0.05);
b) attributes significantly affected by formulation (p ≤ 0.05) and Duncan's test results

The formulation significantly affected other parameters, such as fluidness ($p = 0.013$), adhesiveness to spoon ($p = 0.044$), meltability ($p = 0.005$), and amount of granules ($p = 0.007$). Figure 5.3 showed that the less fluid RUTF was the form.4, followed by form. 3, and the most fluid ones were the form. 1 and 2, however they were all medium fluid. For what concerns their adhesiveness score, an opposite trend was observed, in particular form. 1 and 2 were the less adhesive, followed by form 3 and finally by form 4. Meltability scores showed the same trend observed for fluidness and also the score was quite similar. Finally amount of granules only distinguished form. 1 from the others, in fact in form 1 only few granules were found (3 on 10 scale), meanwhile in the other formulation presented more granules (5 on 10 scale). Adhesiveness to the spoon seems to follow the same trend of consistency index. Panellists are not able to distinguish differences among samples for granule dimensions, meanwhile laser diffraction particle size analyser did. From non-oral sensory evaluation, it was underlined that formulations differed for amount of granules more than for granules dimension. Fluidness, meltability and adhesiveness are sensory attributes that could all affect the difficulty in swallowing, crucial for this type of product.

5.2.4.2. Preliminary consumer tests

Based on the results of non-oral sensory evaluation, consumers evaluated the intensity of odor, sweetness, overall flavor, difficulty in swallowing, graininess, aftertaste, and also the overall liking.

Results showed that the Q^2 cumulated index was high with two components (0.926 and 0.959 for the first and the second component, respectively). This suggested that the quality of the fit was globally good. The 2 components generated by the Partial Least Squares regression summarized well both the Xs and the Ys.

The correlations map (**Figure 5.4.**) allowed visualizing on the first two components the correlations between the Xs and the components, and the Ys and the components.

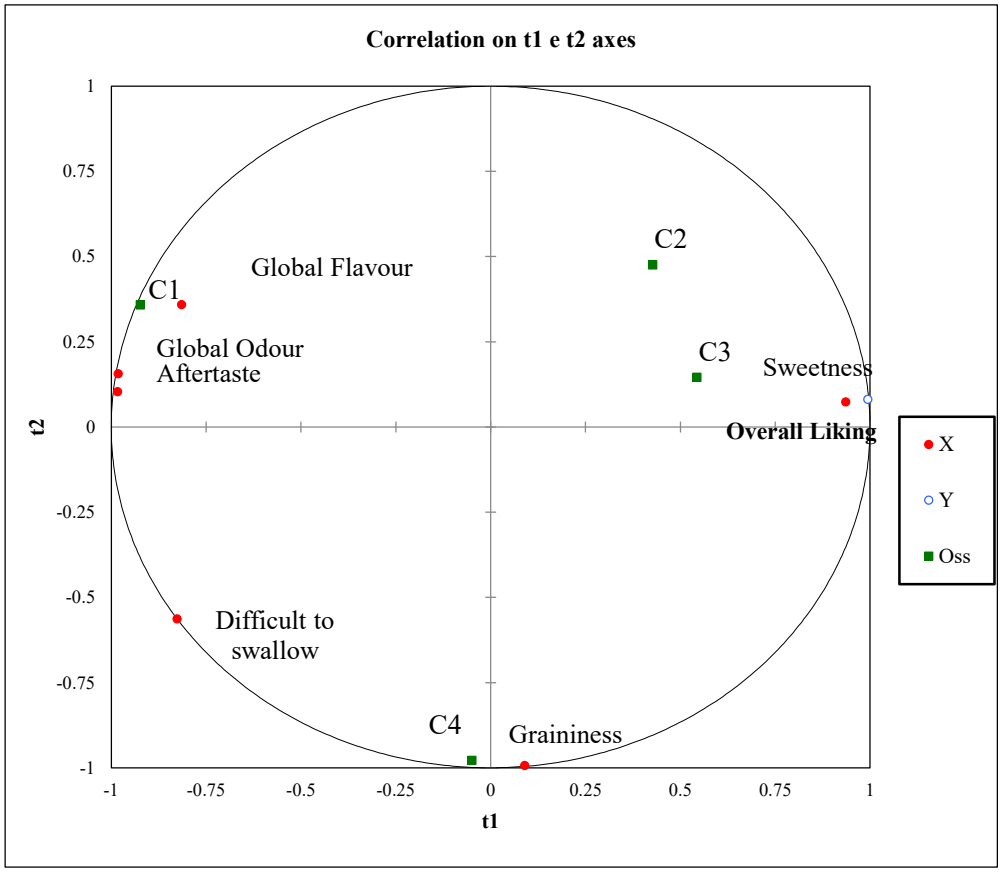

Figure 5.4. Partial Least Squares regression plot

PLS Data observations
Data matrix (X): perceived intensity of the evaluated sensory attributes
Data matrix (Y): overall liking
Dipendent vectors: four formulations

The overall liking was positively correlated to the sweetness and negatively to global flavour, odour, aftertaste and difficult to swallow.

Sample 1 was characterized by sweetness and appeared as the most liked sample. Sample 2 and 3 were similar to each other and were characterized by

high intensity of overall flavour, odour and aftertaste. Finally, sample 4 was perceived grainy and the most difficult to swallow.

Regarding the explanatory variables, the sweetness and the odour/flavour attributes were well represented on the first dimension. This can be interpreted as the fact that these variables explained well the liking of the consumers, which is not surprising as it has a strong effect on taste or other criteria that could easily influence the consumer's preferences. Graininess and difficult to swallow were well represented on the second dimension and there was a good correlation between them.

These results are in according to the composition of the samples. Indeed, samples 1 and 4 had the same concentration of soy flour but different concentrations of oil, that explains the high correlation with the global flavour, odour and aftertaste. However, the formulation 4 contained less oil, that explains why it was more related to the graininess. Finally, formulations 2 and 3 were more related to the sweetness, because they contained more sugar than the other formulations.

5.3. Conclusion

Sensory characterization was a useful tool because it allowed to draw attention to key attributes for children, sweetness and difficulty in swallowing. However, formulations were not so much different from each other and were medium liked by consumers. From those results it was possible to choose the best RUTF for a future clinical trial.

5.3. References

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6. Nutritional Analysis on Alternative RUTF final formulas and Plumpy' Nut ®

6.1. Materials and Methods

6.1.1. Sample preparation

The adopted ingredients, as we reported in the paragraph 1.2, were dehulled soy, dehulled sorghum, sugar, sunflower oil, soy lecithin and Arthrospira maxima (Spirulina) dried powder. The treatments, carried out on raw materials, were defined in order to obtain powders suitable for the cream production process. For soy and sorghum, it was necessary to roast, to reduce the water activity and to degrade the anti-nutritional components. For these reasons, the thermal history defined was 120°C for 3 hours in a fluid bed dryer. Subsequently, the grinding step was optimized for both the roasted seeds and the dried Spirulina in a PM-200 Ball Miller (Retsch, Germany). Raw roasted seeds were initially grinded at 1 mm cut off, then at 250 µm; Spirulina directly at 250 µm. The purpose of scaled up production was to emulate local technological conditions, without availability of transformed ingredients such as flours. For cream production, a stirred ball mill was used (model Micron 20, Selmi s.r.l, Modena, Italy). 60 kg spheres of 5.5 mm diameter were used. Ingredient weight was set on 10 kg, according to manual specifications of the instrument. Speed rate was fixed (72 rpm) and the temperature was controlled (32 ± 3°C). Refining time was 240 min for all the formulations.

6.1.2. Technological characterization (Water Activity)

a_w of 4 alternative RUTF and Plumpy' Nut ® samples was performed as already described in *3.1.1.3* subparagraph.

6.1.3. Fatty Acid Methyl Esters

Fatty acids methyl esters (FAMEs) of the microalgae were produced according to Christie (1989), slightly modified. Oil samples (repeated in triplicate; n = 3) were diluted in hexane (1% oil) and 0.4 mL solution was added to 0.2 mL methanol solution with 2N KOH. The mixture was shaken vigorously for 1 min and the hexane organic phase was collected for GC injection. A Shimadzu model GC-17A equipped with FID (Shimadzu Italy, Milan) was used for the analysis. The acquisition software was Class-VP Chromatography data system version 4.6. (Shimadzu Italy, Milan). A capillary column SPTM-2560, 75 m, 0.14 μm i.d., with 0.18 mm poly (biscyanopropyl siloxane) (SUPELCO, New Heaven, U.S.A.) was used.

The oven temperature was held at 200°C for 5 min and then it increased at a rate of 2° C/min until 230 °C, held for 30 min. Injector temperature and FID temperature: 240°C. Carrier gas: Helium. Column flow: 0.3 mL/min. Split ratio: 1/100. Injected volume: 2 mL. Peak identification was performed by comparing the retention times of the fatty acids with those of pure compounds (mixture of pure methyl esters of fatty acids; Larodan, Malmoe, Sweden) injected under the same conditions.

6.1.4. Lipid quantification

NutriMax creams were analyzed for the quantification of the total lipid fraction by a modified method compared to that proposed by **Bligh & Dyer** in 1959 (Bligh and Dyer, 1959).

Each sample, in triplicate, was weighed, after which it was subjected to a series of steps to facilitate the extraction of the lipid fraction, avoiding losses of lipophilic material during the whole process.

Five grams of each replication were mixed with 20 mL of a 2:1 chloroform / methanol solution, after which they were stirred for 20 minutes on the tilting

plane and centrifuged for 10 minutes at 4000 rpm. The supernatant was recovered, while the remaining pellet was subjected twice to the same steps described above. The total supernatant was treated with 50 mL of an aqueous solution of 0.5% sodium chloride, in order to separate the lipid phase from the hydrophilic phase by means of a separating flask. Following a further and appropriate dehydration with sodium sulfate (two spoons), the lipid phase was inserted into a flask (of which the empty weight was recorded) and dried with a rotating vacuum evaporator (Rotovapor).

The percentage of the lipid fraction in the replication was obtained by the formula:

$$\frac{Balloon\ weight\ with\ extacted\ matter\ (g) - Empty\ balloon\ weight\ (g)}{Sample\ weight\ (g)} \times 100$$

6.1.5. Protein quantification

NutriMax creams were analyzed about the protein fraction using the **AOAC 990.03** method.

Each sample, in triplicate, was weighed, after which it was sent to the mineralization process, typical of Kjeldahl, in the presence of 20 mL of 98% sulfuric acid and 1.5 g of potassium sulphate / copper sulphate (4.5/0.5 p/p) at the temperature of 400 °C, for 3 hours. Ammonium sulphate, formed by the nitrogen mineralization, has been neutralized and, at the same time, distilled in the distiller available in the laboratory. In this way, the ammonium was converted into ammonia which, in turn, was retrotitulated by 0.5 N sodium hydroxide by an excess of 0.5 N sulfuric acid, in the presence of four drops of methylene blue indicator / methyl red (1g/2g in 1000 mL of MetOH 96%). At this point, the amount of inorganic nitrogen was transformed into protein nitrogen by an appropriate conversion factor: *6.25*.

In equation:

$$Total\ sample\ proteins = \left(\frac{H_2SO_4 Volume\ retrotitulated\ by\ NaOH \times H_2SO_4\ Normality \times Nitrogen\ e.w.}{1000}\right) \times 6{,}25$$

Where H_2SO_4 Volume retrotitulated by NaOH = initial mL H2SO4 0,5 N – used mL NaOH 0,5 N - (initial mL H2SO4 0,5 N of the blank – used mL NaOH 0,5 N to titulate the blank).

6.1.6. Total Dietary Fiber

The total fiber was measured using the **AOAC 985.29** gravimetric protocol. Three phases were followed: preparation of crucibles, enzymatic digestion and determination of total fiber.

a) *Preparation of the crucibles*

The crucibles were washed with deionized water, acetone and, again, deionized water through a vacuum flask connected to a pump. Subsequently, 0.5 grams of celite were weighed, added to the crucible and washed with deionized water to allow the formation of a celite filter on the surface of the crucible. Each crucible was then left in muffle at 505 °C overnight.

b) *Enzymatic digestion*

Each sample was prepared in triplicate. One gram of each replica was suspended in 40 mL of 0.05 mM TRIS-MES buffer at pH 8.2 (prepared by mixing 19.52 g of MES [2 (N-morphino) ethanesulfonic acid] and 14.20 g of TRIS [Tris (hydroxymethyl) aminomethane], bringing the solution to pH 8.2 through NaOH 6 N and arriving at a volume of 2 L with deionized water). Each replica was incubated for 35 minutes at 100 °C in the presence of 50 µL of α-amylase, with the aim of obtaining hydrolysis and depolymerization of the starch. Subsequently, after having lowered the temperature to 60 °C, 100 µL of proteases were added, with the purpose of solubilizing and depolymerizing the proteins, for a time equal to 30 minutes. Afterwards, the reaction was

stopped by bringing the pH between 4.1 and 4.8 by adding 0.546 M HCl and the aid of a pH meter. Finally, 200 µL of amyloglucosidase was added to the solution which remained in incubation at 60 °C for further 30 minutes, with the purpose of hydrolysing the surviving starch and glucose fragments.

c) *Determination of total fiber*

At the end of the steps described above, about 300 mL of hot ethanol (60 °C) were added to the hydrolyzed solution of each replica and left in extraction for one hour, with the aim of precipitating the soluble fiber and eliminating the proteins and the de-polymerized glucose. The fiber was obtained by filtering the ethanol solution through a system consisting of the crucible (previously prepared) connected to a suction pump. At this point, the residue deposited on the filter was treated, in sequence, by 3 washes with 25 mL of 78% ethanol, 2 washes with 10 mL of pure ethanol and 2 washes with 10 mL of acetone.

Each of the replicas was prepared in duplicate.

The two crucibles of each of the three replicas were placed in the Memmert oven (Germany) at 103 ° C overnight.

The next morning the two crucibles were weighed, calculating, on the basis of the tare of the crucible, the weight of the dry residue obtained by eliminating the water.

One of the two crucibles was placed in the flask at 505 °C for five hours, in order to obtain the weight of the ashes, while the other crucible was used to determine the quantity of proteins by the Kjeldahl method (described above).

The Total Dietary Fiber (TDF) was calculated using the following equation:

$$TDF\ \% = \frac{\left\{\left[R - \left(p + \frac{A}{100}\right) \times R\right] - B\right\}}{M} \times 100$$

Where:
M = weight of the sample;
R = weight of the dry residue of M;
A = weight of the ashes of M;
p = protein weight calculated from R;
B = blank.

White has been calculated as follows:
B = BR - BP - BA
Where:
BR = dry blank residue;
BA = blank ash;
BP = blank proteins.

6.1.7. Ashes

The total ash was calculated according to the standards contained in the **AOAC 923.03** method. Each sample was analyzed in triplicate.

To perform this experiment, ceramic capsules were used, capable of withstanding very high temperatures, since in the muffle it reaches 505 ° C. Each capsule (used for each replica) was previously placed overnight in the flask at 505 °C, after which it was weighed empty. At this point, the weighed sample (5 g) was added and incineration was started overnight at 505 °C. The next morning, to complete the operation, 10% nitric acid (2 mL) was added, after which the capsule was left at the same temperature as above for another night. The next day, finally, the system was left to cool and the capsule weighed with the ashes.

The percentage of ashes is given by the following equation:

$$Ashes\ \% = \frac{Ashes\ capsule\ weight\ (g) - Empty\ capsule\ weight\ (g) - Blank}{Sample\ weight\ (g)} \times 100$$

Where
Blank = Empty capsule weight + Evaporated nitric acid (2 mL).

6.1.8. Dry Matter

NutriMax creams were analyzed for the quantification of total moisture, following the standards contained in the **AOAC 935.29** method. Each sample was analyzed in triplicate.

Each metal capsule (used for each replica) was first left in the Memmert furnace (Germany) at 103 ° C for a whole night, after which it was weighed empty. At this point, the sample was inserted inside (previously weighed, 2g per replica) and the whole was placed in the oven for another night at 103 ° C. Finally, the weighing of the dry residue was carried out.

The percentage of water in the sample is given by the following equation:

$$R.U.\% = \frac{Dry\ Matter\ Capsule\ Weight\ (g) - Empty\ Capsule\ Weight\ (g)}{Sample\ Weight\ (g)} \times 100$$

6.2 Results and discussion

NutriMax optimized creams showed protein profiles quite similar to the Plumpy' Nut ® one (**Figure 6.1.**). In particular, the fourth was the highest protein content formulation, similar to the first one; the second and the third formulations were very similar in protein content. The first and the fourth NutriMax formulations were higher in protein content than the Plumpy' Nut ®, while the second and the third lower. This is explained by a lower soybean content in the second and third NutriMax formulation than the first and the fourth ones.

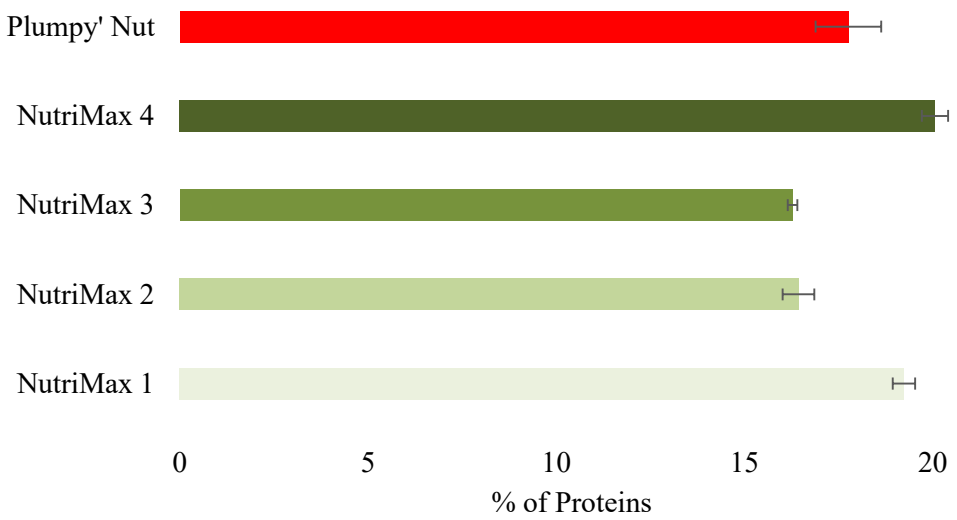

Figure 6.1. Protein profile of the four NutriMax optimized formula and of the Plumpy' Nut ®.

Plumpy' Nut ® can be used as a reference for RUTF protein content (Santini et al., 2013). From this point of view, NutriMax formulations were all suitable for child consumption, because of upper than the range suggested for this type of products by UNICEF and FAO (**Chapter 2**).

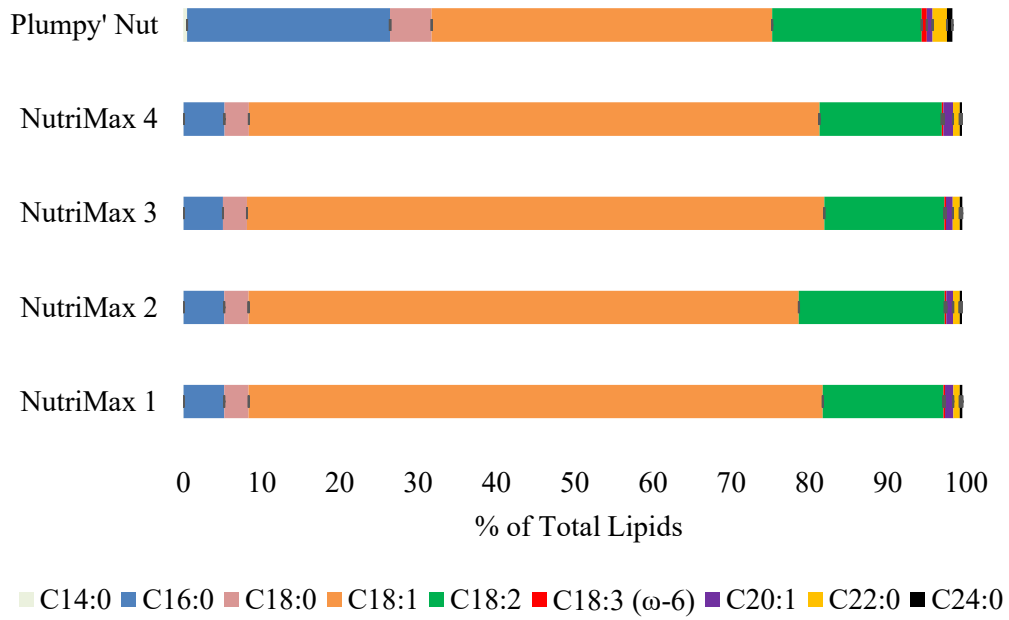

Figure 6.2. Fatty Acid Methyl Esters profile of the four NutriMax optimized formula and of the Plumpy' Nut ®.

Figure 6.2. shows the Fatty Acid Methyl Esters (FAMEs) profile of the NutriMax optimized formulations and of the Plumpy' Nut ®. As reported in the ingredient declaration of the commercial RUTF (**Chapter 2**), Plumpy' Nut ® was made also by a consistent amount of palm oil (in blue, in the figure 6.2.), instead of sunflower oil adopted for NutriMax. Another important characteristic was that in NutriMax formulations the amount of oleic acid was much more than that of Plumpy' Nut ® (in orange, in the figure 6.2.). This aspect reflected on the technological consistency of NutriMax, that appeared much more fluid than Plumpy' Nut ® that was plastic and almost solid (**Chapter 3**). In general, all NutriMax optimized formulations appeared similar in FAMEs composition, with a similar lipid nutritional contribute.

Regarding the *nutritional chart* of the NutriMax optimized formulations, compared with Plumpy' Nut ®, the results are reported in **Table 6.1.** (at page 103).

All NutriMax optimized formulations were very poor in water content (R.U.%), with values two times lower than the Plumpy' Nut ® one. This was an important aspect both regarding microbial stability and the energy and nutritional densities. The water activity of the NutriMax formulations was similar to Plumpy' Nut ® one. On the other hand, Total Dietary Fiber of NutriMax formulations was two times higher than Plumpy' Nut ® amount, but however under the limit of 5% suggested by WHO and FAO to avoid micronutrient losses. Protein, lipid and carbohydrate amounts of NutriMax formulations were different among single production recipes: the first and the fourth formulations were much higher in proteins and lipids than the second and the third; and much lower in carbohydrates than the second and the third.
An important consideration is that, watching about WHO, FAO and UNICEF specifications (**Chapter 2**), **the second and the third NutriMax optimized formulations appeared much more equilibrate** and aligned than the first and the fourth ones to the actual international standards.

Table 6.1. Nutritional Chart of the 4 optimized NutriMax formulations and of the Plumpy' Nut ®.

	NutriMax 1		NutriMax 2		NutriMax 3		NutriMax 4		Plumpy Nut ®	
	Mean value (n=3)	St.Dev.	Mean value (n=3)	St.Dev.	Mean value (n=3)	St.Dev.	Mean value (n=3)	St.Dev.	Mean value (n=3)	St.Dev.
Proteins	**19.242**	± 0.294	**16.443**	± 0.421	**16.281**	± 0.126	**20.063**	± 0.347	**17.767**	± 0.873
Fats	**45.365**	± 0.557	**43.836**	± 0.298	**43.513**	± 0.525	**45.027**	± 0.503	**35.104**	± 0.982
Carbohydrates*	**27.530**	/	**32.938**	/	**33.787**	/	**27.478**	/	**38.839**	/
Total Dietary Fiber	**4.414**	± 0.295	**4.013**	± 0.137	**3.911**	± 0.055	**4.076**	± 0.048	**2.188**	± 0.061
Ashes	**2.307**	± 0.099	**1.731**	± 0.025	**1.489**	± 0.008	**2.206**	± 0.012	**3.937**	± 0.096
Water (% R.U.)	**1.142**	± 0.059	**1.040**	± 0.071	**1.020**	± 0.041	**1.150**	± 0.050	**2.167**	± 0.006
Water Activity (aw)	**0.35**	± 0.09	**0.41**	± 0.07	**0.34**	± 0.06	**0.32**	± 0.04	**0.31**	± 0.08

* Carbohydrates were calculated by difference of the other components.

6.3. References

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7. Conclusions and future perspectives

This PhD-Thesis ends with some important results regarding the analysed development of the alternative Spirulina-based RUTF (called NutriMax).

First, **NutriMax was rheologically suitable for human consumption**, because of its granulometry and viscosimetry. In particular, the second and the third optimized formulations were particularly appreciated as a compromise between the range of the design adopted for this study.

Second, **NutriMax was resistant, at high environment temperatures (until 60° C), to oxidative processes**. In particular, the second and the third optimized formulations were chemically stable, for at least 15 days, a period that permits the production and the distribution in place, without complicate packaging strategies.

Third, **NutriMax was very appreciated under a sensorial point of view**. The second and the third optimized formulations were particularly palatable, with a very good sweetie taste. This result suggests that, in future and local acceptability trials, NutriMax could be largely appreciated by malnourished children.

Fourth, **NutriMax was complete under a nutritional point of view**, following the technical specifications for RUTF formula suggested by WHO, FAO and UNICEF. In particular, the second and the third optimized formulations were nearer to Plumpy' Nut ® nutritional balanced composition than the others.

Fifth, **NutriMax was cheaper than Plumpy' Nut ®, especially under the point of view of the ingredient cost**s (that cover almost the half of total production costs). This issue could be very interesting to solve, in the regional markets, the problem of higher selling prices of the actual locally produced commercial RUTF than offshore produced RUTF. NutriMax could be a valid

cheap alternative for locally produced RUTF market, with a lot of benefits in the perspective of diffusion of this technology.

Nevertheless, other work is requested to complete the planning and the adoption of NutriMax recipe and technology. In fact, first of all**, is necessary to build a small scale production plant, directly in place, to start acceptability and clinical trials**. Second, on the other hand, is very important to **test the protein and lipid digestibility of NutriMax spread**, to be sure of effectiveness in the treatment of protein loss illnesses, such as wasting, stunting and oedema.

At the end, I founded, almost two years ago, a Voluntary Association called "**NutriAfrica**", to collect the money for small scale local plant building. In particular, thanks to a collaboration between Univeristy of Naples and Gulu University (Uganda), will be possible to build the plant there, in Gulu. Nowadays, we collected 25,000 Euros, but the target is 50,000. Our motto is: "**We don't try it. We do it**."

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"Fata volentem ducunt, nolentem trahunt." (Seneca)

"*Un vincitore è semplicemente un sognatore che non si è mai arreso.*" (Nelson Mandela)

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