UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULTY OF PHARMACY

MARIANA WANESSA SANTANA DE SOUZA

DEVELOPMENT AND CHARACTERIZATION OF ENTERAL NUTRITION FORMULAS WITH FUNCTIONAL INGREDIENTS AND PROBIOTIC *Bifidobacterium longum* BL 05

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Thesis presented to the Postgraduate Program in Food Science of the Faculty of Pharmacy of Universidade Federal de Minas Gerais, as a partial fulfillment of the requirements to obtain the Doctoral Degree in Food Science.

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Co-advisor: Prof. Dra. Raquel Linhares Bello de Araújo

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DESENVOLVIMENTO E CARACTERIZAÇÃO DE FÓRMULAS PARA NUTRIÇÃO ENTERAL COM INGREDIENTES FUNCIONAIS E PROBIÓTICO Bifidobacterium longum BL 05

MARIANA WANESSA SANTANA DE SOUZA

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To Good,

to my family and my teachers.

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Life is generous and with every living room,
so many other doors are discovered.
And life enriches those who risk opening new doors.

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ABSTRACT

The aim of this study was to evaluate the effect of inulin, medium-chain triglycerides (MCT) and whey protein isolate (WPI) on enteral nutrition formulas (ENF) and the influence of these ingredients on physicochemical parameters, protein *in vitro* digestion and functionality of bifidobacteria incorporated to the product. For this, a *centroid simplex* experimental design was used, including a central point and a control formula. Firstly, the formulas were characterized for chemical composition, emulsion stability and *in vitro* protein digestibility. In a second phase, the effect of the three ingredients on the viability of *Bifidobacterium longum* BL05 was evaluated during the product storage, for 120 days, at 4 ^{o}C, as well as the survival of the microorganisms under simulated gastrointestinal conditions. Overall, eight formulas were developed, presenting mean values of 17.3% of protein, 62.3% of carbohydrates, 11.5% of lipids, a caloric value of 420 kcal/100 g, all nutritionally adequate according to the Brazilian regulation. The emulsion stability of the suspended formulas was affected by all ingredients and the interactions between MCT – inulin, and MCT – WPI contributed positively to the improvement of this parameter. Regarding the protein digestibility, the presence of inulin promoted lower percentages of degree of hydrolysis and small peptides. *B. longum* BL 05 counts ranged from 9.05 to 9.79 log CFU g^{-1}, and the WPI showed a positive effect on the viability at the end of the storage period. The presence of inulin exerted a protective effect on cells when subjected to simulated gastrointestinal conditions, with log reductions between 2.03 and 3.44 log CFU g^{-1} after 4 hours of simulated digestion. Thus, the developed formulas demonstrated adequacy regarding the composition and physicochemical parameters, as well as a potential food matrix for the incorporation of probiotic microorganisms.

Keywords: Product development. Experimental design. Stability. Digestibility. Probiotics survival.

RESUMO

O objetivo deste trabalho foi avaliar o efeito da inulina, dos triglicerídeos de cadeia média (TCM) e da proteína isolada de soro de leite (WPI) em fórmulas para nutrição enteral (FNE) e a influência destes ingredientes nos parâmetros físico-químicos, na digestão *in vitro* das proteínas e na funcionalidade de bifidobactérias incorporadas ao produto. Para isso, foi utilizado um delineamento experimental *centroid-simplex*, incluindo um ponto central e uma fórmula controle. Em primeiro lugar, as fórmulas foram caracterizadas quanto à composição química, estabilidade da emulsão e digestibilidade *in vitro* das proteínas. Numa segunda etapa, avaliou-se o efeito dos três ingredientes na viabilidade de *Bifidobacterium longum* BL05 durante o armazenamento do produto, durante 120 dias, a 4 ^{o}C, bem como a sobrevivência dos micro-organismos sob condições gastrintestinais simuladas. Ao todo, oito fórmulas foram desenvolvidas, apresentando valores médios de 17,3% de proteína, 62,3% de carboidratos, 11,5% de lipídios, valor calórico de 420 kcal/100 g, todas nutricionalmente adequadas de acordo com a regulamentação brasileira. A estabilidade da emulsão das fórmulas foi afetada por todos os ingredientes e as interações entre TCM – inulina e TCM – WPI contribuiram positivamente para a melhoria deste parâmetro. Em relação à digestibilidade proteica, a presença de inulina resultou em menor liberação de pequenos peptídeos e aminoácidos livres. As contagens de *B. longum* BL 05 variaram entre 9,05 a 9,79 log UFC g-1, e o WPI apresentou um efeito positivo sobre a viabilidade no final do período de armazenamento. A presença de inulina exerceu um efeito protetor nas células quando submetidas a condições gastrointestinais simuladas, com reduções entre 2,03 e 3,44 log UFC g^{-1} após as 4 horas de digestão simulada. Assim, as fórmulas desenvolvidas demonstraram adequação quanto à composição e parâmetros físico-químicos, bem como uma potencial matriz alimentar para a incorporação de microrganismos probióticos.

Palavras-chave: Desenvolvimento de produto. Delineamento experimental. Estabilidade de emulsão. Digestibilidade *in vitro.* Sobrevivência de probiótico.

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ACRONYMS

ANOVA	Analysis of variances
ANVISA	Agência Nacional de Vigilância Sanitária
AOAC	Association of Official Analytical Chemists
a_w	Water activity
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CD	Crohn's Disease
CFU	Colony-forming unit
cP	Centipoise
Da	Dalton
DH	Degree of Hydrolysis
DS	Droplet size
DVS	Direct Vat Set
EFSA	European Food Security Authority
EN	Enteral nutrition
ENF	Enteral nutrition formulas
FAO	Food and Agriculture Organization
GRAS	Generally Recognized as Safe
GIT	Gastrointestinal tract
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
LCT	Long-chain triglycerides
MCT	Medium-chain triglycerides
MPN	Most Probable Number
MRS	de Man, Rogosa & Sharpe
NAFLD	Nonalcoholic Fatty Liver Disease
NURC	Nonspecific Ulcerative Retocolitis
OPA	Ortho-phthalaldehyde
O/W	Oil-in-water
PPGCA	Programa de Pós-Graduação em Ciência de Alimentos
RDC	Resolução da Diretoria Colegiada

RPM	Revolutions Per Minute
RSM	Response Surface Methodology
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SE-HPLC	Size-Exclusion High Performance Liquid Chromatography
SCFA	Short-Chain Fatty Acids
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SSS	Simulated Salivary Solution
SGS	Simulated Gastric Solution
SIS	Simulated Intestinal Solution
TEC	Total Energy Content
UFMG	Universidade Federal de Minas Gerais
USP	United States Pharmacopeia
WHO	World Health Organization
WPC	Whey Protein Concentrate
WPI	Whey Protein Isolate

CONTENTS

1 INTRODUCTION

The use of nutritional therapy has been growing significantly, since the evidence shows that the nutritional status directly influences the clinical evolution of the patient, besides reducing the prevalence of infections and complications, mortality rates, time and hospitalization costs (BRASIL, 2016; WAITZBERG *et al*., 2017; CORREIA; LAVIANO, 2018*).*

Among the nutritional therapy forms, Enteral Nutrition (EN) is indicated for those patients who are unable to supply at least 60% of their nutritional needs and for those who present the gastrointestinal tract fully or partially functional (PEIXOTO, 2015; BRASIL, 2016). In this scenario, the industrialized enteral nutrition formulas (ENF) are widely used and according to the Brazilian legislation, these must meet the composition requirements and must contain proteins, lipids, carbohydrates, vitamins and minerals (BRASIL, 2015).

Optionally, enteral nutrition formulas may contain fibers such as inulin, a non-digestible glucose polymer, that has been widely used in enteral formulas because it does not significantly interfere with the physical characteristics and stability of food (JAKOBSEN *et al*. 2017). Also, it has the property of modulating the intestinal microbiota and can exert a protective effect on microorganisms during the storage of probiotic foods, as well as during passage through the gastrointestinal tract (BURITI *et al*., 2010; COSTA, 2014). Whey proteins are of high biological value, have functional and bioactive properties and are indicated as a protein source for enteral formulas (ABRAHÃO, 2012; SILVA *et al*., 2014). Medium-chain triglycerides (MCT) are used by the enteral route as a source of lipids because of their ease of absorption and vegetable oils of coconut, babassu and palm kernel are considered as good sources of these fatty acids (WHO, 1999; MARTEN *et al*., 2006; PEIXOTO, 2015).

Also, given their beneficial health potential, probiotics are foreseen as optional ingredients for enteral formulas and are defined by FAO/WHO (2002) as “living microorganisms which, when administered in adequate amounts, confer health benefits to the host". Among these benefits, we highlight the modulation of the intestinal microbiota, protection against pathogens, improvement of the immune system and therapeutic applications in clinical conditions such as irritable bowel

syndrome, ulcerative colitis and infectious diarrhea (SAAD *et al*., 2013; SANDERS *et al*., 2018).

Traditionally, probiotics are consumed in fermented products, but new foods have been evaluated as vehicles for these microorganisms (CHAMPAGNE *et al*., 2011). However, the incorporation of probiotics into an appropriate food matrix depends on several factors such as processing and storage conditions, in addition to food composition, pH, water activity and the presence of additives (TRIPATHI; GIRI, 2014). Therefore, it is important to evaluate the viability of the bacteria and also their resistance to gastric acid and bile salts, when incorporated in the food (BURITI *et al*., 2010).

The food industry faces a constant challenge in the development of new foods, intending to combine the achievement of quality products, with affordable costs and meeting the expectations of consumers. For this purpose, statistical methods can be used to determine the optimum levels of the main ingredients, optimizing the ideal responses for the physicochemical, rheological and sensory parameters (CASTRO *et al*., 2003; GRANATO; CALADO, 2011). When evaluating the influence of several factors on the final result of food, the experimental design of mixtures, called *centroid simplex,* has been used to propose a new formulation *(*BARROS NETO *et al*., 2007; GRANATO; CALADO, 2011).

Thus, the aim of this study was to develop formulas for enteral nutrition containing inulin, medium-chain triglycerides and whey protein isolate and to evaluate the influence of these ingredients on physical parameters and *in vitro* digestibility of proteins. Additionally, we evaluated the viability of *Bifidobacterium longum* BL 05 during the storage of ENF, its survival under *in vitro* gastrointestinal conditions and the influence of inulin, MCT and whey protein isolate on these parameters.

2 OBJECTIVES

The main objective of this work was to evaluate the influence of inulin, medium-chain triglycerides and whey protein isolate on physical parameters, *in vitro* digestibility of proteins and on the viability and survival of probiotics in enteral nutrition formulas.

2.1 Specific objectives

- Develop enteral nutrition formulas (ENF) using a *centroid-simplex* design.
- Determine the proximate composition and the viscosity of the ENF developed.
- Evaluate the emulsion stability by measuring the droplet size and zeta potential.
- Evaluate the *in vitro* protein digestibility by the degree of hydrolysis and the peptide profile.
- Determine the pH and the water activity of the formulas.
- Incorporate the freeze-dried probiotic *Bifidobacterium longum* BL05 into the formulas.
- Evaluate the probiotic viability during 120 days of refrigerated storage of the formulas.
- Evaluate the probiotic survival under *in vitro* simulated gastrointestinal conditions.
- Determine the microbiological quality of the enteral formulas produced.

3 LITERATURE REVIEW

3.1 Enteral nutrition

The term Enteral Nutrition (EN) comprises all forms of nutritional support involving the use of foods for special purposes, intending to maintain or recover the nutritional status of the patient, performed in people unable to adequately meet their nutritional and metabolic needs orally. This includes oral supplements, as well as feeding via nasogastric, nasoenteral or percutaneous tubes (PEIXOTO, 2015; ROSENFELD, 2019).

The EN is indicated to patients with a fully or partially functioning gastrointestinal tract and malnutrition or risk of malnutrition, that is when the oral intake is less than 60% of the energy needs. Besides when the patient cannot, should not or does not want to feed by the mouth, as in cases of unconsciousness, oral injuries, strokes, trauma or severe depression (BRASIL, 2016; TUNKAY *et al*., 2018; CARDOSO *et al*., 2019).

In Brazil, malnutrition represents the most important risk factor for death in institutionalized elderly people, especially those over 75 years old (FERREIRA *et al*., 2011), with an estimated prevalence of up to one in three patients. In the hospital environment, malnutrition is one of the most relevant public health problems, both in industrialized and developing countries, since nutritional deficits are related to increased mortality, morbidity, hospitalization time and the number of re-admissions, as well as high medical-hospital costs. The prevalence of intra-hospital malnutrition may reach up to 50%, depending on the population evaluated and the methods used (BRAZIL, 2016; CORREIA *et al*., 2017; WAITZBERG *et al*., 2017; CORREIA; LAVIANO, 2018).

The main objectives of the EN are to prevent and treat nutritional deficiencies and to prepare the patient for the surgical and clinical procedure. The implementation of adequate nutritional support for critical patients is considered crucial, since adequate protein and energy intake are related to a lower mortality and reduction in the prevalence of complications and infections (BRASIL, 2016; WAITZBERG *et al*., 2017; TUNKAY *et al*., 2018; CARDOSO *et al*., 2019).

Chronic or acute diseases can alter food intake by different mechanisms and the medications, surgery, chemotherapy and radiotherapy can cause side effects

such as loss of appetite, nausea, vomiting and impairment of nutrient absorption, which may promote a nutritional deficiency (WAITZBERG *et al*., 2017; TUNKAY *et al*., 2018).

Also, psychosocial conditions like age, inability to eat alone and depression may favor the reduction in food intake (FERREIRA *et al*., 2011; WAITZBERG *et al*., 2017).

The use of early enteral nutrition not only provides the necessary macro and micronutrients but also provides the functional integrity of the body's largest immune organ, the intestine. Through this therapy, the maintenance of intestinal integrity, immune function and diversity of the gut microbiota is achieved (SAVINO, 2018; ROSENFELD, 2019).

Therefore, it is fundamental to implement early enteral nutrition therapy in patients at risk, and one of the recommended forms is the oral supplementation that, in a systematic review, demonstrated to reduce hospital stay and costs (FREIJER *et al*., 2014; WAITZBERG *et al*., 2017; TUNKAY *et al*., 2018).

3.1.1 Enteral nutrition formulas

The enteral nutrition formulas (ENF) can be industrialized or handmade, the latter being prepared with *in natura* food, minimally processed, conventional food products and/or nutritional modules (HENRIQUES *et al*., 2017; SAVINO, 2018). They usually have a lower cost when compared to the industrialized formulas, but the used ingredients and its preparation can cause some insecurity regarding the nutritional composition and physical-chemical stability (HENRIQUES *et al*., 2017).

In developed countries, the use of industrialized formulas is more frequent, and in Brazil the consumption of this type of formula has been increasing gradually, especially because they are more practical, nutritionally complete and offer greater safety, regarding microbiological control and composition (RIBOLDI *et al*., 2011; HENRIQUES *et al*., 2017; CARDOSO *et al*., 2019; ROSENFELD, 2019).

The industrialized enteral formulas can be presented in three forms: powder for reconstitution, liquid semi-ready (open system) and liquid ready for use (closed system). The advantages of powder formulations are the possibility of individualization of prescription, physical-chemical and microbiological stability, thus

providing micronutrients in appropriate amounts and its storage being facilitated due to small volume. However, when compared to the liquid formulas, it has the disadvantage of needing more manipulation and preparation time (PEIXOTO, 2015; CARDOSO *et al*., 2019; ROSENFELD, 2019).

The Brazilian legislation establishes specific composition and quality requirements for enteral formulas, defining parameters regarding macro and micronutrients. The ENF is defined as *"industrialized food for special medical purposes, suitable for tube use and, optionally, orally, consumed only under medical or nutritionist orientation, specially processed or designed to be used exclusively or complementary in the feeding of patients with limited capacity to ingest, absorb or metabolize conventional foods or patients with specific nutritional needs determined by their clinical condition*" (BRASIL, 2015).

The products covered by this Regulation (RDC 21/2015) must meet the composition requirements and must contain proteins, lipids, carbohydrates, vitamins and minerals by following the amounts and specifications established. In addition, enteral nutrition formulas may be added with dietary fibers, taurine, carnitine, inositol and also probiotics, provided that its safety of use is assessed prior to marketing (BRASIL, 2015).

Standard ENF are those whose composition reflects the reference values for macro and micronutrients of a normal population, that is, they are normocaloric (0.9 to 1.2 kcal/mL), normoproteic (between 10 and 20% of total energy content - TEC), normolipid (between 15 and 35% of TEC) and normoglycidic (between 45 and 75% of TEC), and present the proteins in intact form (polymeric). However, according to the clinical needs for which it is intended, the formulas may undergo changes in their composition, with adjustments in caloric density, the proportion between macronutrients and the addition of optional ingredients, such as probiotics (BRASIL, 2015; CARDOSO *et al*., 2019).

Thus, diets containing beneficial components to the intestinal mucosa, immunomodulating ingredients or capable of reducing the phase of acute inflammation are of great value in clinical practice. These specialized formulas, which contain specific nutrients such as omega 3 fatty acids, probiotics, prebiotics and nucleotides have shown promising results (ARRUDA; AGUILAR-NASCIMENTO, 2004; SAVINO, 2018; ROSENFELD, 2019).

Currently, some of the fiber-enriched formulations contain non-digestible carbohydrates such as oligosaccharides and inulin, which provide beneficial effects, but without changing the physical characteristics or stability of formulations (RAVAT *et al*., 2019). These oligosaccharides are known as prebiotics and promote the modulation of the intestinal microbiota and prevent the exacerbated growth of pathogenic bacteria. Besides, short-chain fatty acids are produced as a by-product of their fermentation and are a substrate for colonocytes (RHA *et al*., 2010; JAKOBSEN *et al*., 2017; TUNKAY *et al*., 2018; WATSON *et al*., 2019).

3.2 Product development and experimental design

The food industry faces a constant challenge regarding new product development, in order to obtain quality products, with affordable costs and meeting consumer expectations. According to Jousse (2008) for both industry and academia, the main points to be considered are the choice of variables and the optimization of the main desirable characteristics of the food. In this way, the industries have been using statistical methods to determine the optimum levels of the main ingredients and thus obtaining optimal responses from physicochemical, rheological and sensory parameters (CASTRO *et al*., 2003; GRANATO; CALADO, 2011).

The first step in the product development is the choice of the main ingredients, having as premises the understanding of its functionalities and nutritional properties, as well as its possible interactions. It is important to know the current legislation, for the adequate use of types and concentrations of the many ingredients, in addition to its possible functional properties (EARLE *et al*., 2001; HORVAT *et al*., 2019). Preliminary tests are then required to evaluate the minimum and maximum values of the main ingredients in order to obtain the desired characteristics, followed by statistical techniques in the evaluation of the result (GRANATO; CALADO, 2011).

When evaluating various factors (ingredients or process conditions) in the development of a product, several statistical methods can be applied. Some are simpler, such as those with only two factors and two levels, like 2^2 factorial planning, or others that are more complex, as the central composite, depending on the objective (CRUZ *et al*., 2010). When a new formulation or new food is proposed, the most appropriate planning is the Mixture Design, which it is possible to determine the

optimal composition of each component in the mixture in order to achieve a product with the best characteristics (GRANATO; CALADO, 2011; MANFIO; LACERDA, 2016).

The experimental design involving mixtures of ingredients differs from the other types of designs since the properties of the final product are defined by the ratio between the ingredients and not by the absolute values. The sum of the proportions of the ingredients in a mixture is always 100%, and if a modification of the properties of this mixture is required, the proportion between its components shall be changed, but always respecting its totality. Due to this specificity, experimental design methods have been modified to adapt to the specific problems of mixtures, and these methods are widely used, including in the food industry (BARROS NETO *et al*., 2007).

In the case of food, most formulations consist of ternary mixtures, which are formed by three components, and in the experimental design, these are considered as three independent factors. In Figure 1 it can be observed that these are represented by *X1*, *X2* and *X3*, which must add 100% and correspond geometrically to an equilateral triangle, where the vertices are equal to the totality of only one of the components, the sides to mixtures of the equivalent components on that side, and the central point to the sum of all factors (CASTRO *et al*., 2003; BARROS NETO *et al*., 2007; COSTA, 2014).

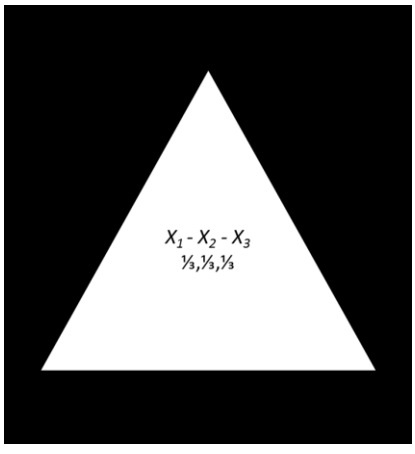

Figure 1 Spatial representation of formulations according to *simplex centroid* design for mixing modeling.

(Adapted from Barros Neto *et al*., 2007).

The experimental design commonly used in the case of ternary mixtures, is called *centroid simplex*, a derivation of the Response Surface Methodology (RSM), which is a set of techniques based on factorial design and the use of the “minimum square method”, adjusted for the construction of empirical models, describing the behavior of the system under study, based on the responses obtained (BARROS NETO *et al*., 2007; COSTA, 2014). It has been widely used in foods systems such as dairy beverages (CASTRO *et al*., 2003; OLIVEIRA *et al*., 2018); enteral formula (BUENO, 2008a; BUENO, 2008b); *petit suisse* cheese (CARDARELLI *et al*., 2008); mousse type dessert (BURITI *et al*., 2010); fruit juices (HAMINIUK *et al*., 2011); ice cream (COSTA *et al*., 2016) and fruit peel flour (DANESI *et al*., 2018).

Optimization in food development is a way of achieving optimal process conditions to obtain the desired quality (GUPTA; BAJAJ, 2017; OLIVEIRA *et al*., 2018). When more than one response is relevant to the product´s final result, a graphical overlay approach can be used for the different response surfaces obtained, thus finding the experimental region that provides the desired values for each response (GRANATO *et al*., 2010). This approach, called desirability function, is very promising for the optimization of several answers, besides being easily executed employing specific software (DERRINGER; SUICH, 1980 CRUZ *et al*., 2010; HORVAT *et al*., 2019).

3.3 Ingredients

The ENF must meet the requirements of composition and quality and must contain proteins, lipids, carbohydrates, vitamins and minerals, with quantities and characteristics defined by the specific legislation. Regarding proteins, they must be from animal or plant, in their intact or hydrolyzed form and meet minimum values of essential amino acids, according to the reference protein, and within this requirement, whey proteins are widely used (ABRAHÃO, 2012; BRASIL, 2015, SAVINO, 2018).

For lipids, the current regulation establishes levels between 15 and 35% of the total energy value, in addition to specific quantities of fatty acids such as lauric and myristic, mono and polyunsaturated, highlighting the use of medium-chain triglycerides due to their greater ease of absorption (BRASIL, 2015; LEWIS; ABREU, 2017; NAGASAKA *et al*., 2018).

In addition to the essential components, ENF may be added with nutrients such as dietary fibers, taurine, carnitine and inositol, also at levels previously defined in the resolution (BRASIL, 2015; SAVINO, 2018; ROSENFELD, 2019).

3.3.1 Inulin

Prebiotics are used in foods as functional ingredients because they have the ability to modulate the gut microbiota. They are defined as “the substrate that is selectively utilized by host microorganisms conferring a health benefit” (GIBSON *et al*., 2017).

Among these ingredients can be highlighted the inulin, which belongs to the group of fructans, and is a glucose and fructose polymer, linked by β-(2-1) bonds, with degree of polymerization between 2 and 60, average size of 12 monomeric units (Fig. 2) (GUIMARÃES *et al*., 2018; VERRUCK *et al*., 2019). It has moderate solubility in water, low viscosity, is naturally found in foods such as onion, artichoke, asparagus, banana and leek, and industrially is extracted from the chicory root (AL-SHERANI *et al*., 2013; REZAEI *et al*., 2014).

This type of binding present in the fructans, in general, is resistant to hydrolysis by mammalian enzymes and to the intestinal absorption however, the prebiotic fermentative action is given by the specificity of the bifidobacteria in producing intracellular inulinase, an enzyme capable of hydrolyzing such bonds. The final products of fermentation are short-chain fatty acids (SCFA), such as propionate, butyrate and acetate, which are responsible for several beneficial functions attributed to probiotics. Additionally, the presence of SCFA reduces intraluminal pH, increasing the solubility of calcium and magnesium salts, favoring their absorption (SOUSA, 2013; RAY, 2018; TEIMOURI *et al*., 2018).

It should be noted that due to fermentation by colonic bacteria, prebiotics, including inulin and all non-digestible oligosaccharides, provide approximately 1,5 kcal/g (ROBERFROID, 1999).

In addition to its biological effects, inulin has important technological properties such as improving the viability of probiotic cultures, improving the texture of food products, as well as can be used as a fat substitute, acting as an emulsifier/stabilizer (GUIMARÃES *et al*., 2018).

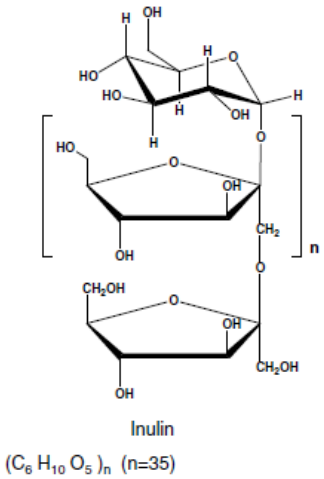

Figure 2 Chemical structure of inulin.
Source: MANSO *et al*., 2008.

The incorporation of inulin, associated with the presence of probiotics, in order to obtain symbiotic foods, has been evaluated in several dietary matrices, such as ice cream, fermented milk, yoghurt and *petit suisse* cheese (CARDARELLI *et al*., 2008; BURITI *et al*., 2010; SOUZA, 2010; SOUSA, 2013; REZAEI *et al*., 2014; COSTA *et al*., 2016; SANTOS *et al*., 2019; OZTURKOGLU-BUDAK *et al*., 2019).

The use of inulin is foreseen in the Brazilian legislation as dietary fiber, and for enteral formulas is allowed the presence of up to two grams per 100 kilocalories of formula ready for consumption (BRASIL, 2015).

3.3.2 Medium-chain triglycerides (MCT)

Lipids are a heterogeneous group of biological compounds not soluble in water (hydrophobic) and soluble in organic solvents (such as ether and chloroform). This group includes oils, fats, some vitamins and hormones, as well as components of cell membranes. The composition of the lipids present in the food consists mainly of triglycerides, resulting from the esterification of a glycerol molecule with three fatty acids (FUENTES, 2011; SAVINO, 2018).

The term medium-chain triglycerides (MCT) corresponds to the mixture of the triglycerides composed of the fatty acids with 6 to 12 carbons in their chain, that is, of

the caproic, caprylic, capric and lauric acids (MARTEN *et al*., 2006; SILVA, 2013). MCTs have different chemical and physiological properties when compared to long-chain triglycerides (LCTs), since they are smaller and more water-soluble molecules (0.68 mg/mL for C8:0 and 0.72 mg/mL for C10:0), which increases its solubility in biological fluids. Therefore, these properties affect the way they are absorbed and metabolized (SHAHIDI, 2006; NAGASAKA *et al*., 2018).

In the intestinal lumen, MCT hydrolysis is faster and more efficient than LCTs, and consequently, the absorption of medium-chain fatty acids is more efficient, since most of them are transported through the portal vein to the liver, whereas long-chain fatty acids are incorporated into kilomicrons and are transported by the lymphatic system (MARTEN *et al*., 2006). Unlike LCTs, medium-chain fatty acids pass easily through the cytoplasmic membrane and are transported directly to mitochondria without the mediation of L-carnitine (NAGASAKA *et al*., 2018).

Because of their greater ease of absorption, MCTs are widely used in the clinical area in enteral and parenteral nutrition formulations, especially for patients in conditions of malabsorption. Additionally, they are commonly included in enteral formulas because they are easily digested and are related to the improvement of hepatic and immunological functions (SILVA, 2013; SAVINO, 2018).

Among the oils rich in MCTs and widely used in the food industry, we can highlight the oils of coconut (*Cocos nucifera*), babaçu coconut (*Orbignya* ssp.) and palm kernel (*Elaeis guineenses*) and the latter is obtained from the palm fruit. This oil compared to coconut has a softer odor and taste and the fatty acid profiles are very similar (WHO, 1999).

According to the *Codex Alimentarius*, palm kernel oil has a composition of between 45 and 55% lauric acid, 2.6 and 5% of capric acid and 2.4 to 6.2% of caprylic acid. The manufacture of palm kernel oil for human consumption requires refining, consisting of neutralization, bleaching, filtration and deodorization (JIN el., 2008).

Besides the nutritional importance, the incorporation of different types of lipids, such as MCT, can influence technological characteristics, as well as the functionality of probiotics present in foods (SANTOS *et al*., 2018).

3.3.3 Whey proteins

Whey is composed mainly of water (93%), lactose (4.5-5.0%), soluble proteins (0.6-0.8%), lipids (0.4-0.5%) and mineral salts (8.0-10.0%). Quantitatively, the whey proteins account for around 20% of total bovine milk proteins and consist basically of β-lactoglobulin, α-lactoalbumin, bovine serum albumin, immunoglobulins, in addition to lactoferrin, lactoperoxidases and glycoproteins (ABRAHÃO, 2012; GUIMARÃES *et al*., 2019).

Liquid whey is a sub-product of the dairy industry of relative importance since it is produced in large quantities and has a high nutritional quality, especially due to its lactose and soluble protein content. However, this is still considered as an agro-industrial waste, especially in countries such as Brazil being improperly disposed, and causing serious environmental problems due to its high content of organic matter (SILVESTRE, *et al*., 2012; PINTO *et al*., 2015; GUIMARÃES *et al*., 2019).

Besides its widely described nutritional properties, whey presents high concentration of branched-chain amino acids, especially leucine and isoleucine, which are related to increased muscle tissue, with tissue regeneration in multiple traumas, in addition to improvement in the inflammatory profile, which is of great interest in patients using enteral formulas (ABRAHÃO, 2012, SAVINO, 2018). Whey proteins are also valued for their technological functional properties, when used as ingredients in food formulations, especially due to their solubility and emulsifying capabilities (PAGNO *et al*., 2009; GUIMARÃES *et al*., 2019).

The use of this protein source in enteral formulas is more indicated than casein, since whey proteins have faster digestion and absorption, with shorter gastric emptying time, thus reducing the possibility of complications, such as aspiration or gastroparesis (SAVINO, 2018). In a clinical trial conducted in 2009, enteral formulas with different protein sources were compared (casein, hydrolyzed soy, whey protein concentrated and hydrolyzed whey protein) in rats with atrophied intestinal mucosa by the use of prolonged parenteral nutrition. The results showed that whey proteins were able to reverse the hypoplasia of the mucosa, unlike casein and soya, besides promoting weight gain (ABRAHÃO, 2012).

The use of whey proteins as a food ingredient in the industry is mainly by the concentrates (Whey Protein Concentrate - WPC), which are products obtained from different filtration processes and with protein contents between 25 and 80%. While

whey protein isolates (WPI) are those with protein contents above 90% and very low fat and lactose content (COSTA, 2014; ZIEGLER, 2016).

3.4 *In vitro* protein digestibility

Food digestion is a complex, dynamic and essential process for human health. The release and absorption of nutrients will be used by the body for growth, maintenance of tissues and as an energy source. This process involves the mechanical transformations, capable of reducing the particle size and the action of enzymes, in which macromolecules are hydrolyzed and absorbed, reaching the bloodstream. The first stage occurs mainly in the mouth and stomach, while the enzymatic action occurs primarily in the small intestine (RAMSAY; CARR, 2011; MINEKUS *et al*., 2014).

To deepen the knowledge about the processes and influence of various parameters on human digestion, several experimental models, both *in vitro* and *in vivo*, have been developed in recent years (GUERRA *et al*., 2012). However, the simulation of a physiological and physicochemical mechanism of such complexity involves the use of realistic steps in terms of time, pH and enzymatic conditions. In this sense, the use of *in vitro* protocols presents as advantages the execution time and relatively smaller costs, besides avoiding *in vivo* models, which are extremely expensive and invasive (MINEKUS *et al*., 2014; MAT *et al*., 2018).

Various *in vitro* digestion methods and protocols, with different stages, incubation conditions, types of enzymes and electrolyte concentration, have been employed, which makes the direct comparison between the results obtained by different authors difficult. Therefore, a harmonised *in vitro* enzyme digestion protocol established by more than 200 scientists, standardising oral, gastric and intestinal phase parameters, was published in 2014, on the basis of an international consensus, defining a static model accessible and easy to apply by the scientific community (MINEKUS *et al*., 2014; EGGER *et al*., 2016; MAT *et al*., 2018).

This harmonised protocol consists of three stages. In the oral phase, first occurs the reduction of the food particle size and the addition of the simulated salivary solution (SSS). In the mouth, the texture of the solid food is significantly altered by chewing and salivation, with the formation of a cohesive food bolus and ready to be swallowed. The protocol recommends that the oral phase should be

performed even for liquid foods, especially if they contain a high concentration of glucose polymers. The SSS is composed of several electrolytes, such as sodium, calcium, potassium, magnesium, phosphates and bicarbonate, in addition to α-amylase. The amount of SSS should be sufficient to form a paste and the recommended incubation time is two minutes (MINEKUS *et al*., 2014).

In the simulated gastric digestion stage, the main objective is the cleavage of proteins by the action of pepsin in an acid medium (pH 3.0), for two hours. Similarly, a simulated gastric solution (SGS) containing electrolytes is employed at concentrations similar to the gastric content. Finally, despite being many variables that impact on intestinal transit time, the protocol determines that the intestinal phase is also performed for two hours, with neutralization of the medium to pH 7.0 and addition of simulated intestinal solution (SIS), bile salts and pancreatin, an enzyme complex with proteolytic, amylolytic and lipolytic action (MINEKUS *et al*., 2014).

In this way, *in vitro* digestion experiments have been widely used to evaluate the bioaccessibility of nutrients and non-nutrients, as well as the digestibility of macronutrients, especially proteins and lipids. Since the structure and composition of the food can influence the release and consequent metabolism of nutrients, the understanding of which and how these factors can interfere in the kinetics of the digestive process becomes of fundamental importance (MINEKUS *et al*., 2014; MAT *et al*., 2016).

Proteins are the most important macronutrients in food. Their digestion is performed by the action of pepsin in an acid medium in the stomach and by pancreatic and duodenal enzymes in the intestinal phase. Normally, studies to evaluate the digestibility of proteins are conducted with them in solution, while the proteins in our diet are present in solid or semi-solid foods, and even in liquid form, tend to precipitate and form a solid mass in the stomach. Therefore, it is important to evaluate the influence of the food matrix on the protein digestibility (LUO *et al*., 2015; MAT *et al*., 2016).

Several methodologies can be employed to evaluate the extent of protein digestibility. The determination of the Degree of Hydrolysis (DH), can be by measuring the α-amino nitrogen (MAT *et al*., 2016; MAT *et al*., 2018) or by ortho-phthalaldehyde (OPA) derivatization (LUO *et al*., 2015; HEJAZI, ORSAT, 2016; RUI *et al*., 2016; MULCAHY *et al*., 2017). Besides the distribution of peptides, according to molecular mass, such as electrophoresis and chromatographic methods

(ISKANDAR *et al*., 2015; LUO *et al*., 2015; NGUYEN *et al*., 2015; EGGER *et al*., 2016; RUI *et al*., 2016).

DH is defined as the percentage of cleaved peptide bonds relative to the total number of peptide bonds in the protein. The methods are based on the determination of the free α-amino groups, the nitrogen released during hydrolysis or the titration of the protons released by cleavage of the peptide bonds (MORAIS *et al*., 2013; MAT *et al*., 2016). Among the most widely used techniques, the determination of the α-amino groups is highlighted by the use of OPA as a derivatizing agent (LUO *et al*., 2015; HEJAZI; ORSAT, 2016; MAT *et al*., 2016; RUI *et al*., 2016; MULCAHY *et al*., 2017).

Ortho-phthalaldehyde (OPA) is a fluorogenic agent capable of reacting with organic molecules containing primary amino groups (-NH_2), in the presence of a reducing agent and in alkaline medium. The method currently employed is based on the determination of the derivatives formed by the reaction of the OPA with the α-amino groups released during hydrolysis, in the presence of 2-mercaptoethanol, at 340 nm (CHURCH *et al*., 1983; MORAIS *et al*., 2013).

The advantages of this test justify its wide use in the assessment of protein digestibility, such as solubility and stability of the reagent in an aqueous medium, speed, possibility of performance at ambient temperature, sensitivity and low cost per test (MORAIS *et al*., 2013).

Besides the determination of the DH, it is important to evaluate the peptide profile, according to the molecular weight, in order to analyze the impact of the *in vitro* digestibility process on protein degradation. The chromatographic methods and gel electrophoresis techniques have been widely used (MORAIS *et al*., 2013; ISKANDAR *et al*., 2015; LUO *et al*., 2015; NGUYEN *et al*., 2015; EGGER *et al*., 2016; RUI *et al*., 2016). The characterization of the peptide profile is also relevant given that oligopeptides, especially di- and tripeptides, are more effectively absorbed than an equivalent mixture of free amino acids, thus representing an advantage from a nutritional point of view (MORAIS *et al*., 2015).

When assessing the digestion of WPI and egg white proteins, both in solution or in a gel form, Luo *et al*. (2015) observed that the release of free amino acids was lower for both proteins, when they were in a gel form. Being in solution, the WPI presented a degree of hydrolysis of 15.0%, while when in gel, its digestion reached only 7.9% DH. According to these authors, in high viscosity dietary matrices, the proteins are immobilized in the colloidal network, avoiding the action of the proteolytic

enzymes. The peptide profile, assessed by molecular exclusion chromatography, was also used as a means of assessing the extent of protein digestibility, and similarly, the release of peptides between 4.0 kD and 2.0 kD and less than 2.0 kD were more significant when proteins were in solution.

3.5 Emulsion stability

The enteral formula, after dispersion in water, is a type of oil in water (O/W) emulsion, a heterogeneous system, consisting of at least one immiscible liquid dispersed in another liquid in the form of droplets. Being an unstable system, their stability may be affected by substances such as surfactants or emulsifiers (ROLAND *et al*., 2003; McCLEMENTS, 2005; McCLEMENTS, 2007; LOPES, 2010). Non-polar molecules tend to be located in the oily phase of the emulsion, the polar ones, in the aqueous phase and those with polar and apolar characteristics at the interface (CASTEJON, 2010; LOPES, 2010; ZIEGLER, 2016).

Among the ingredients used in the formulation of the present study, the soybean lecithin, as well as the whey protein isolate, act as emulsion stabilizers, because they have hydrophilic and lipophilic groups in the same molecule (SUI *et al*., 2017).

The emulsions stability can be analyzed by visual observation of the phase separation and by the droplet size or the change (zeta potential) of the fat particles in the dispersed phase (HENRIQUES; ROSADO, 1999; ARAÚJO; MENEZES, 2006; VON ATZINGEN *et al*., 2007; CASTAGNARO *et al*., 2013).

3.5.1 Droplet size

The emulsion stability is strongly influenced by the size of the particles that compose it, and can directly influence changes such as gravitational separation, flocculation or coalescence. Therefore, it is essential to accurately and reliably evaluate the size of the particles present in the emulsion (McCLEMENTS, 2007; ALMEIDA, 2012).

When in an emulsion, the oil and water droplets present the same size, this can be considered as monodisperse and the radius or diameter of the droplet can be

used to characterize the emulsion. However, generally, dietary emulsions are considered to be polydispersed, since they present a variety of sizes, given their heterogeneous characteristics of composition and thus, is best characterized by a droplet size distribution, commonly represented by a histogram or by the concentration of particles in each percentile size category (WALSTRA, 2003; McCLEMENTS, 2007; BARBOSA *et al*., 2009; ALMEIDA, 2012).

To determine the characteristics of all the particles present, it is of fundamental importance to know the full distribution of particle size, which can thus relate to the possible origin or nature of any system instability. When the loss of stability occurs by gravitational separation or flocculation there is no change in the size of each drop, while when it occurs through coalescence, there is an increase in the average droplet size (McCLEMENTS, 2007; ALMEIDA, 2012). However, for control purposes, it is more convenient to use the particle size distribution in the emulsion, and a central trend measurement, such as mean, median and relative standard deviation (WALSTRA, 2003; McCLEMENTS, 2007; ALMEIDA, 2012).

Many analytical techniques can be used to determine particle size in emulsions, most of which are automated, providing fast and reliable measurements. Such methods differ according to the physical principles on which they are based, such as light scattering, particle speed in a field, dispersion or adsorption of ultrasonic waves, particle count (ALMEIDA, 2012).

Laser diffraction is currently one of the most widely used techniques for determining droplet size, being a fast, easy to perform and adaptable method for samples in different physical states. It is based on the diffraction phenomenon that occurs between a laser beam that focuses on the particles, which is characteristic for each particle size. Thus, a mathematical model accurately provides the particle size distribution profile of the analyzed sample (USP, 2005; BARBOSA *et al*., 2009).

3.5.2 Zeta potential

The potential existing between the surface of a particle and its associated ions is called zeta potential (ζ), and its measurement is a very useful tool in the evaluation of repulsive interactions between colloidal particles, because it is directly

related to emulsion stability (LAOUINI *et al*., 2012; LOPES *et al*., 2013; LOPES, 2014).

According to Laouini *et al*. (2012) when one of the three states of matter – solid, liquid or gaseous, is dispersed in another, we have a colloidal system, most of them having electric charges on their surface, being related to the chemical nature of the particle components and the environment itself. The generation of these charges occurs mainly through the ionization of the surface groups (negative by the acid groups and positive by the basic ones) and the adsorption of charged species to the surface of particles (LAOUINI *et al*., 2012; LOPES, 2014).

For the measurement of emulsion stability, high zeta potential values, both negative and positive, indicate that there will be repulsion between the particles, thus reducing their aggregation trend. Therefore, colloidal dispersions with zeta potential values around 30 mV are usually considered stable. On the other hand, low-value particles may not prevent flocculation and such rules cannot be strictly considered, especially in systems containing stabilisers, which decrease the numerical value of the zeta potential due to the change of the particle shear plan (LAOUINI *et al*., 2012; LOPES *et al*., 2013; LOPES, 2014).

The physicochemical stability of lipid emulsions occurs with the use of phospholipids, derived mainly from egg or soy lecithin, which act as emulsifiers. These phospholipids are positioned at the oil/water interface, giving the oil droplets a negative electrostatic charge, resulting in the stabilization of the emulsion and giving rise to the zeta potential. Thus, the measurement of this potential is an effective way of controlling the behavior of the emulsion, since it indicates the relationship between the surface potential and the repulsion forces between the droplets (LAOUINI *et al*., 2012; SUI *et al*., 2017).

The emulsions those zeta potential are between -50 and -30 mV are considered stable. However, in complex formulations containing bivalent electrolytes and cations can reduce the surface tension or destabilize the emulsion by a process of neutralization of the charges, leading to the coalescence (CASTANARO *et al*., 2013).

The method for determining the zeta potential consists of the incidence of a laser beam and the simultaneous application of an electric field by the sample. Thus, charged particles travel at different speeds inducing displacements of incident light beam frequency, generating a frequency spectrum, which are then used for speed

calculations, whereas in turn converted to values of electrophoretic mobilities (MALVERN INSTRUMENTS, 1996; LOPES, 2014).

3.6 Probiotics

The number of bacteria in the human body is of the same order as the number of human cells itself, estimated in 10^{13}. The microbiota is established in various parts of the human body, such as skin and gastrointestinal tract, where it has an important impact on human health (SOUZA *et al*., 2012; LEE *et al*., 2014; SANZ, 2016; SENDER *et al*., 2016; RAJOKA *et al*., 2017).

The microbiota present in the digestive tract represents an essential component to the metabolic balance of the host, since it acts in the immune system, interacting with lymphoid tissue and intestinal epithelium, promoting the regulation of antimicrobial peptides, gene expression and modulating cellular permeability. Besides, a balanced and healthy microbiota is necessary for the maturation of lymphocytes and the maintenance of adequate levels of immunoglobulins. Thus, disturbances in the interaction between diet, metabolism and microbiota are an important factor in the regulation of the body's homeostasis (SANDERS *et al*., 2013; LEE *et al*., 2014; RAJOKA *et al*., 2017; CHENG *et al*., 2019). However, according to the differences between diet and lifestyle, the microbiota varies widely among individuals. Abusive use of antibiotics, poor diet in fermentable carbohydrates, excessive measures of sanitization, cesarean birth and use of artificial infant formulas are known factors that negatively affect intestinal colonization (DALIRI; LEE, 2015; DERRIEN; VEIGA, 2017; RAJOKA *et al*., 2017).

Therefore, supplementation with specific microorganisms could benefit human health, such as probiotics, that are defined by FAO/WHO as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (HILL *et al*., 2014; KUMAR; SALMINEN, 2016; FLOCH, 2017 RAJOKA *et al*., 2017; RAVAT *et al*., 2019; PRADHAM *et al*., 2020).

The genera mostly studied and used as probiotics are those found at high levels in the human intestine and from healthy animals such as *Enterococcus*, *Lactobacillus*, *Lactococcus* and *Bifidobacterium* (IANNITTI; PALMIERI, 2010; BURNS *et al*., 2014; DALIRI; LEE, 2015; SHORI, 2016; PEREIRA *et al*., 2018; PRADHAM *et al*., 2020).

These genera are commonly recognized as GRAS (Generally Recognized as Safe), given their long history of safe use, with rare cases of bacteremia caused by the translocation of the intestinal lumen into the blood circulation, reported only in severely immunosuppressed patients (PRADHAM *et al*., 2020). Floch (2017) points out that most of the reports of complications related to the administration of probiotics were with the yeast *Saccharomyces boulardii*, especially since it is not a microorganism found in the human gastrointestinal tract.

Some conditions must be met by microorganisms to be considered as probiotic, as safety aspects (origin, pathogenicity, antimicrobial susceptibility); technology (processing resistance and storage); functional (adherence and resistance to gastrointestinal tract conditions) and finally host benefits, as evidenced by placebo-controlled clinical trials (FAO/WHO, 2002; IANNITTI; PALMIERI, 2010; SOUZA *et al*., 2012; SAAD *et al*., 2013; DALIRI; LEE, 2015).

It is emphasized by some authors the importance that the probiotic is of human origin since the microorganism would better perform its functions in an environment similar to that which it was isolated (MORAIS; JACOB, 2006; SZAJEWSKA *et al*., 2006). However, it is also known that other microorganisms such as the yeast *S. boulardii*, although not of human or animal origin, has been used as probiotic since the 1960s, with widely demonstrated results in the prevention and treatment of infectious diarrhea (HTWE *et al*., 2008; SOUZA, 2012). Currently, it is recommended that human origin be a selection criterion for a probiotic microorganism, but not an eligibility condition and that those of non-human origin require further studies to evaluate its safety and efficacy (RANADHEERA *et al*., 2010; PRASANNA *et al*., 2014; FALEIRO, 2015).

Another important factor to be considered is the ability to adhere to the intestinal epithelium, as it is the first step for colonization, even if temporary and fundamental for the microorganism to exert its effects, especially due to modulation of the immune system and by preventing the adhesion of pathogens. However, there are no known probiotics able to settle in the digestive tract, because the resident microbiota prevents colonization. Therefore, the daily consumption of an adequate quantity probiotic is indispensable for its beneficial effects (POURRAJAB *et al*., 2019).

The viability of probiotic microorganisms is highlighted as a prerequisite for their functionality. However, recent studies have suggested that even non-viable

microorganisms or their components can exert beneficial effects after consumption (SOUZA *et al*., 2012; VIEIRA *et al*., 2016). In assessing the ability to protect mice against enteric *Salmonella*, Souza *et al* (2012) observed that even at low counts (<5 log CFU mL^{-1}) the *Bifidobacterium longum* 5^{1A} was effective. Similarly, Vieira *et al* (2016) observed that the same bifidobacteria strain, inactivated by heating at 70 ^{o}C for 20 min, was able to reduce *in vitro* infection by *Klebsiella pneumoniae*.

For inactivated microorganisms were proposed the term “paraprobiotics”, by Taverniti and Guglielmetti (2011) from the observation that dead microbial cells or even cell fractions are capable of promoting health benefits. The mechanisms of action are still unclear, but they seem to involve modulation of the immune system (cellular components could activate immune cells) or by metabolite secretion (ALMADA *et al*., 2016). However, Daliri and Lee (2015) emphasize that living cells might to be more effective than paraprobiotics.

3.6.1 In vitro selection

The viability of a microorganism can be considered as the number of viable cells displayed by a culture under a certain condition (BURNS *et al*., 2014). Functionality is a more complex concept that involves both viability and factors such as resistance to low stomach pH and bile salts, adherence to epithelial cells and immunostimulation capacity. Therefore, the appropriate choice of a microorganism as a probiotic involves its ability to achieve, survive and persist, even temporarily, in the gastrointestinal tract (GIT) (IANNITTI; PALMIERI, 2010; VINDEROLA *et al*, 2011).

During the passage through the digestive tract the main factors that affect the probiotics' viability are the low stomach pH and the presence of the bile salts in the duodenum, causing loss of enzyme activity and structural damage of cell membrane (VINDEROLA *et al*, 2011; BURNS *et al*., 2014; DALIRI; LEE, 2015) Thus, *in vitro* assessment of resistance to these conditions is mandatory as established by FAO/WHO (2002).

However, currently there is no standard protocol for assessing resistance to GIT conditions but several studies have suggested the importance of using saline as a medium to simulate stomach conditions, in addition to maintaining the pH between 1,5 and 3.5 and also the presence of pepsin in the simulated stomach fluid (ANNAN

et al, 2008; VINDEROLA *et al*., 2011; GBASSI; VANDAMME, 2012; BURNS *et al*., 2014; BERNUCCI *et al*., 2017). For the composition of the simulated intestinal fluid, the use of saline, buffered with sodium phosphate, is recommended in the presence of bile salts and pancreatin, at a slightly alkaline pH (8.0) (GBASSI; VANDAMME, 2012; BERNUCCI *et al*., 2017).

The influence of the food matrix to which the probiotic is incorporated is also emphasized. The food itself is an important factor in the functionality attributes of the microorganism since it will also pass through the different barriers of the GIT. In this way, the characteristics and composition of foods such as fat and protein content, type of proteins, pH and certain ingredients such as flavoring, stabilizing and thickeners or functional ingredients, as bioactive compounds to which probiotics may be exposed influence their activity and functionality (RANADHEERA *et al*, 2010; SHORI *et al*., 2016; CHAMPAGNE *et al*., 2018).

Consequently, the formulation of products containing probiotic should aim at the optimization of all these variables in order to improve the efficiency of a given microorganism strain, or at least not adversely affect its performance (RANADHEERA *et al*, 2010; VINDEROLA *et al*., 2011).

3.6.2 *Bifidobacterium genus*

The first line of species belonging to the bifidobacteria group was isolated in 1899 by Henry Tissier at the Pasteur Institute, who described it as microorganisms in the form of rod, not gas producers, anaerobic, gram-positive, non spore-forming, with and without scourges. They are presented in several forms, as curved and short rods and rods in Y form, and in unfavorable environments are pleomorphic. The optimal pH for growth is around 6.0 to 7.0, with no growth below 4.5 - 5.0. As for temperature, bifidobacteria of human origin have an optimum growth of 36 - 37 ºC, while those of animal origin are between 41 - 43 ºC (GOMES; MALCATA, 1999; PRASANNA *et al*., 2014; SANZ, 2016).

Currently approximately 30 species belonging to the genus *Bifidobacterium* are recognized, at least 11 of them of human origin: *B*. *adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B*. g*allicum*, *B. infantis*, *B*.longum, *B. pseudocatenulatum*, and *B. scardovii* (MEILE *et al*., 2008; PRASANNA *et al*.,

2014; PEREIRA *et al*., 2018). The bacteria of this genus are the most frequently used as probiotics since it is considered one of the safest for use in food (MEILE *et al*., 2008; PRASANNA *et al*., 2014; VERRUCK *et al*., 2015; SANZ, 2016).

3.6.3 Health effects and safety of use

The main health benefits attributed to the ingestion of probiotic microorganisms are the maintenance of the gut microbiota, protection against gastrointestinal pathogens, improvement of the immune system, anticarcinogenic activity and vitamin production (SAAD *et al*., 2011; TRIPATHI GIRI, 2014).

The therapeutic applications with the greatest evidence in the literature are those related to gastrointestinal conditions, such as irritable bowel syndrome (IBS), inflammatory bowel diseases (IBD) such as ulcerative retocolitis and pouchitis, infectious diarrhea, necrotizing enterocolitis and colorectal cancer (SAAD *et al*., 2013; SANDERS *et al*., 2016). It should be noted that the beneficial effects reported in clinical trials are highly dependent on the strain (strain-specific) and that the results observed cannot be extrapolated even for the same species studied (CHENG *et al*., 2019).

Irritable bowel syndrome is one of the most prevalent intestinal disorders in developed and developing countries, affecting up to 15% of the adult population and is characterized by recurrent episodes of abdominal pain associated with altered intestinal habits, whether diarrhea or constipation. Two important studies using *Bifidobacterium infantis* 35624 have demonstrated excellent results and this microorganism has been used to control IBS (SANDERS *et al*., 2013; FLOCH, 2017).

Inflammatory bowel diseases refer to two chronic diseases that affect the gastrointestinal tract differently: Crohn's Disease (CD) and Nonspecific Ulcerative Retocolitis (NURC). In NURC inflammation is delimited to the mucosa of the colon, occurring continuously, while CD can affect any part of the intestinal tract, from the mouth to the anus (IANNITI; PALMIERI, 2010). In the treatment of NURC, the combination of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* species and also the *Escherichia coli* nissle 1917 were effective in inducing and maintaining symptom remission. For CD, no consistent effects are observed (SAAD *et al*., 2013; SANDERS *et al*., 2013; FLOCH, 2017).

It was also observed the effectiveness of the use of probiotics in the prevention of pouchitis, a common inflammation that occurs in the ileoanal pouch, formed in intestinal anastomosis surgeries. The estimated incidence of this occurrence is up to 60% (SANDERS *et al*., 2013; FLOCH, 2017) and in a double-blind placebo-controlled study, only 10% of the group using probiotics presented pouchitis, against 40% of the control group (GIONCHETTI *et al*., 2007).

Possibly, the most common use of probiotics in clinical practice is in the control and prevention of infectious diarrhea or associated with the use of antibiotics. Infectious diarrhea is a leading cause of morbidity and mortality in children under 5 years old worldwide and may lead to malnutrition and growth deficits (SANDERS *et al*., 2013). Several studies with different lines of probiotics, including *S. boullardii*, *Lactobacillus rhamnosus* GG among others, have demonstrated the reduction of the time of acute infectious diarrhea by up to 1 day, and up to 40-60% reduction in the frequency of diarrhea associated with the use of antibiotics (GUADALINI, 2011; SAAD *et al*., 2013; FLOCH, 2017).

Nosocomial infections, that is, those acquired due to the permanence in the hospital environment, represent a great public health problem and generate enormous costs, thus requiring preventive measures. Therefore, supplementation with *Lactobacillus* GG in adults and bifidobacteria in hospitalized children has been shown to be effective in reducing such complications (SZAJEWSKA *et al*., 2011; SANDERS *et al*., 2013; FLOCH, 2017).

Liver diseases have also been the object of evaluation of the effectiveness of probiotics, from the observation of significant changes in the microbiota of patients with chronic liver disease, especially in nonalcoholic fatty liver disease (NAFLD) and in the control of hepatic encephalopathy. In the last decade, several studies have demonstrated favorable results of several probiotic strains, such as *L. bulgaris*, *L. plantarum*, *B. longum* and *L. rhamnosus* (DOULBERIS *et al*., 2017; FLOCH, 2017). Studies have also related the consumption of prebiotics and probiotics before liver transplantation with the reduction of the incidence of infectious complications after surgery and improvement in biochemical parameters (SAWAS *et al*., 2015; GRAT *et al*., 2017).

In addition to clinical applications with greater scientific evidence, other fields of activity have been extensively studied in recent years, with emphasis on food allergies (HUANG *et al*., 2016), obesity (MISHRA *et al*., 2016; CHENG *et al*., 2019),

Alzheimer's disease (AKBARI *et al*., 2016), atopic dermatitis (FUCHS-TARLOVSKY *et al*., 2016), blood cholesterol control (DALIRI; LEE, 2015), celiac disease and lactose intolerance (DALIRI; LEE, 2015; PANGHAL *et al*., 2018).

In surgical patients, supplementation with probiotics and symbiotics was demonstrated as effective, in a recent meta-analysis, in the prevention and control of infections at the surgical site, and other infectious complications and reduction of side effects, besides shorter hospital stay (WU *et al*., 2016). Also in a clinical trial conducted in a Brazilian hospital, the authors observed significant reductions in the incidence of infections, length of stay in intensive care unit and mechanical ventilation, when evaluating the use of an early enteral diet associated with probiotic and glutamine supplementation in patients with brain damage (ARRUDA; AGUILAR-NASCIMENTO, 2004).

Whelan and Myers (2010) attribute the safety of the use of probiotics to the fact that most of the strains used are from human origin and to present a long history of use without reports of complications, the incidence of bacteria is extremely low and related to only specific strains. In a systematic review of clinical trials of the use of probiotics in patients using nutritional support, 32 cases of probiotic infections were reported in a universe of 4131 patients (using enteral or parenteral nutrition), evaluated in 53 studies, most of which presented reduced mortality, sepsis and infections.

3.6.4 Technological aspects in incorporating probiotics into food

Initially, probiotic microorganisms were incorporated into the market through yogurts and fermented products and they still represent a large part of this market (CHAMPAGNE *et al*., 2011; SHORI, 2016; PRADHAN *et al*., 2020). However, they are currently being found in several other food matrices, from fruit juices (THAKUR; SHASMA, 2017; DIAS *et al*., 2018), cereals (CHAMPAGNE *et al*., 2011), margarine (SOUZA *et al*., 2017; SANTOS *et al*., 2018), ice cream (COSTA *et al*., 2016; RODRIGUES *et al*., 2019), desserts (BURITI *et al*., 2010), cheeses (VERRUCK *et al*., 2015), chocolate (MAILLARD; LANDUYT, 2008; SILVA *et al*., 2017), dried fruits (MARCIAL-COBA *et al*, 2019) and infant formula (LIU *et al*., 2015).

Despite this, there are some limitations regarding the incorporation of probiotics into dietary (especially non-lactic) matrices, such as the survival of probiotics throughout the shelf life of the products. The viability of microorganisms and an ideal concentration are necessary for the observation of health benefits and it is estimated that 10^9 CFUs of viable cells per 100 g of the product will be sufficient (PANGHAL *et al*., 2018).

According to Tripathi and Giri (2014) values above 10^6 CFU g^{-1} or mL^{-1} in the product are used by the food industry as acceptable to exert beneficial effects. According to Vinderola *et al*. (2000) consumption of 10^8 - 10^{11} CFU/day would be recommended to achieve the proposed benefits, while Liu *et al*. (2015) quotes values between 10^6 and 10^7 CFU/day.

The current Brazilian legislation does not establish a minimum count for a food to be considered as probiotic, but proof of safety and efficacy, regardless of dose (BRASIL, 2018). The European Union already has a minimum number of 10^8 CFU g^{-1} (EFSA, 2010)

In a recent review of Ouwehand (2017) the variability of clinical trial results evaluating dose-response related to probiotic effects was demonstrated, and an absolute value cannot be established, because the daily dose is dependent on each strain, the desired effect and the individual characteristics of the host. However, the author points out that the vast majority of studies with satisfactory results were performed with doses around 10^8 and 10^{11} CFU/dose.

Processing, storage and packaging conditions become crucial for the maintenance of viability until the end of shelf-life proposed for the product. Factors such as probiotic strain, oxygen concentration, volume, pH, acidity, water activity, presence of salt, sugars and other compounds such as bacteriocins or flavoring and artificial colorants can affect the viability of microorganisms (Fig 3.) (MISHRA; MISHRA, 2012; TRIPATHI; GIRI, 2014; PANGHAL *et al*., 2018).

Champagne *et al*. (2018) also emphasize that the presence of probiotics and compounds such as prebiotics in the same dietary matrix can improve the survival of microorganisms during storage and also their resistance in adverse conditions of the gastrointestinal tract. It is, therefore, necessary to characterize specific strains and their relationship with their respective food matrix and composition (MISHRA; MISHRA, 2012; SHORI, 2016).

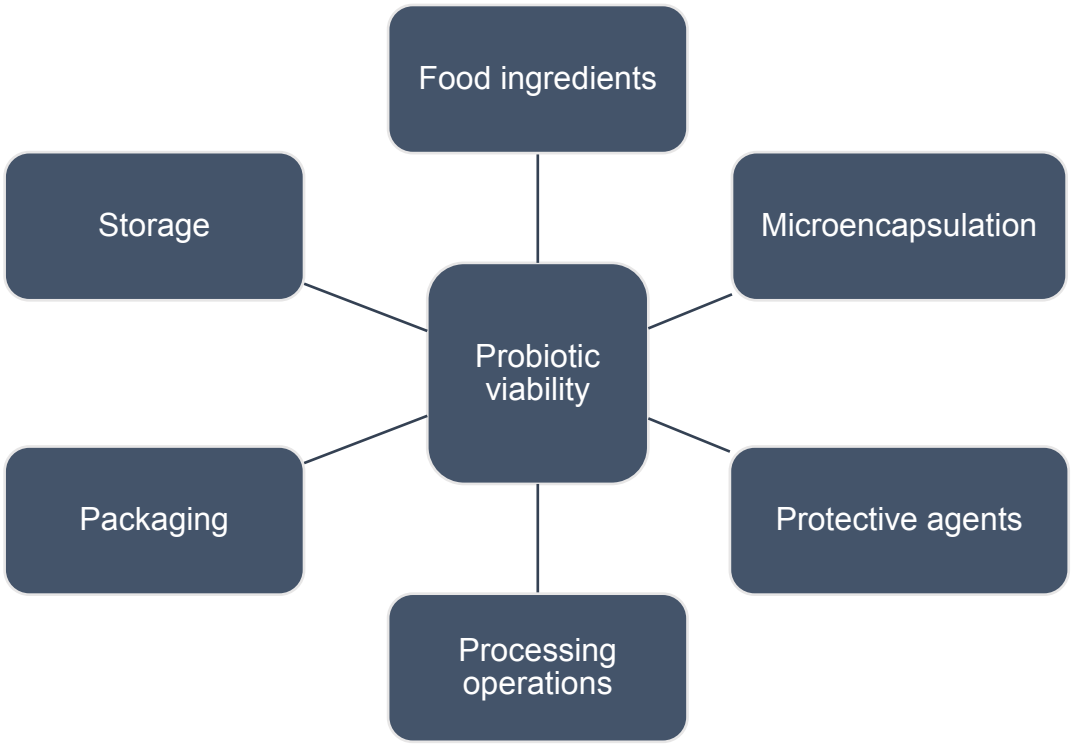

Figure 3 Factors that affect the viability of probiotics in food. Adapted from TRIPATHI; GIRI, 2014.

Evaluating the incorporation of inulin and casein concentrate on the viability of *Bifidobacterium animalis* BB12 in margarine, Souza (2010) noted that these ingredients contributed both to resistance in simulated *in vitro* conditions, as well as maintaining adequate populations throughout storage, when compared to control formulations. Similarly, by incorporating inulin and whey protein concentrate (WPC) into guava mousses, the simultaneous addition of these ingredients was beneficial for the viability of *Lactobacillus acidophilus* (BURITI *et al*., 2010).

Protein concentrates can also improve the viability of probiotic cultures in food matrices due to the presence of proteins and phosphates, which act as buffering agents and inhibitors of digestive protein activity *in vivo* (ANTUNES *et al*., 2005; COSTA, 2014; YASMIN *et al*., 2019). Besides, probiotic microorganisms may be able to hydrolyze proteins, forming peptides and amino acids that are fundamental to the culture itself. Therefore, it is interesting to evaluate the incorporation of this type of ingredient in the formulations (PRASANNA *et al*., 2012; COSTA, 2014; KAREB; AIDER, 2019).

Thus, several authors evaluated the influence of the presence and different concentrations of whey proteins (in the form of WPC or WPI) on the viability of probiotic cultures. Assessing the resistance of *L. acidophilus* to simulated gastric conditions, KOS *et al*. (2000) observed that WPC was more efficient in protecting the microorganism when compared to casein and skimmed milk, increasing from 15% to 45% of the control sample for WPC treatment.

The addition of WPC in guava mousses improved the viability of *L. acidophilus* La-5 and gave a protective effect to the microorganism when subjected to simulated gastrointestinal conditions (BURITI *et al*., 2010). In a study conducted by the same research group, it was evaluated the addition of WPC in ice cream with symbiotic activity containing inulin, in the protection of *L. acidophilus* and *B. animalis* subsp. l*actis*. They showed improvement of technological, sensory and resistance characteristics in *in vitro* tests (SOUSA, 2013).

According to Wada and Lonnerdal (2014), bifidobacteria are quite demanding and require specific growth factors, and the proteins present in whey protein concentrates (β-lactoglobulin and α-lactoalbumin) are excellent promoters for these microorganisms.

In a study with probiotic yogurts, the incorporation of WPC and fruit-oligosaccharides in the viability of *Bifidobacterium animalis* was evaluated, and the supplementation of 1.5% of whey proteins increased the viable count organisms in a logarithmic cycle after the first week of storage, when compared to the control product, without addition of proteins (AKALIN *et al*., 2007).

The storage conditions and the human gastrointestinal tract are hostile for probiotic microorganisms, but the food matrix can protect the cells, avoiding great reductions in probiotic populations (SOUZA et al., 2017). Thereby, the food matrix, its compositions and physicochemical properties play an important factor in probiotic strains (CHAMPAGNE *et al*., 2018).

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CHAPTER I

EFFECT OF INULIN, MEDIUM-CHAIN TRIGLYCERIDES AND WHEY PROTEIN ISOLATE ON STABILITY AND *IN VITRO* DIGESTIBILITY OF ENTERAL NUTRITION FORMULAS

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EFFECT OF INULIN, MEDIUM-CHAIN TRIGLYCERIDES AND WHEY PROTEIN ISOLATE ON STABILITY AND *IN VITRO* DIGESTIBILITY OF ENTERAL NUTRITION FORMULAS

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ABSTRACT

This study aimed to evaluate the impact on digestibility and physical properties of enteral formulas by the addition of variable ingredients. Eight formulas were designed by a *simplex-centroid* evaluating different concentrations of inulin, medium-chain triglycerides (MCT) and whey protein isolate (WPI). Overall, the eight formulas developed presented mean values of 17.3% of protein, 62.3% of carbohydrates, 11.5% of lipids, a caloric value of 420 kcal/100 g, thus obtaining nutritionally adequate formulas. The emulsion stability of the suspended formulas was affected by all ingredients and the interactions between MCT – inulin, and MCT – WPI were effective in improving this parameter. Besides that, the use of inulin mostly affected the protein digestibility, according to the degree of hydrolysis and the peptide profile. The desirability function (*d*-value = 0.769) proposed a formulation containing 0.70% of inulin, 1.56% of MCT, and 1.73% of WPI. This proposed solution may improve enteral formulas because this has optimal emulsion stability and protein digestibility, which are essential characteristics for a product to be used by patients under special clinical conditions.

Practical application: Stability and protein digestibility of enteral formulas were affected by ingredient replacement.

Keywords: Enteral formula; emulsion stability; *in vitro* gastrointestinal digestion; design of experiments.

1 INTRODUCTION

Enteral formulas are administrated for patients who are unable to consume conventionally food due to an illness condition. Many formulas have been developed to supplement nutrients to patients with these conditions. Also, they show prebiotic and pharmacological potential properties (Anvisa, 2015; Brown, *et al*., 2015; Pei *et al*., 2019; Zhuang *et al*., 2019). The digestibility and bioaccessibility are strongly influenced by their structure and composition (Mat *et al*., 2016; Simsek *et al*., 2017; Azzollini *et al*., 2018).

The nutrient absorption and digestion are two of the main criteria of enteral formula effectiveness. It can be measured by assessing some of their properties, such as digestibility and stability. These may be considered good indicators of their quality and potential consumer acceptance (Aguilera, 2018; Li *et al*., 2019).

The structure and composition of foods can significantly influence macronutrient digestibility of nutrients, and little is known about the mechanisms of this process. *In vitro* digestion protocols have been widely used for this evaluation and are relatively faster and less expensive than *in vivo* models (Minekus *et al*., 2014; Luo *et al*., 2015; Mat *et al*., 2016; Simsek *et al*., 2017).

Stability is directly related to the food matrix. It can be measured by assessing the complex assembly of nutrients and non-nutrients and how they interact. Chemical and physical variation in formulas influences their properties such as accessibility and digestibility (Aguilera, 2018). Enteral formulas are produced with standard ingredients or may contain specific added or replacing ingredients, aiming to improve its effectiveness (Savino, 2018; Portela *et al*., 2019).

Medium-chain triglycerides (MCT) are substances with 8 – 12 carbons which are absorbed faster than other types of lipids (Kinsella *et al*., 2017). They may reduce infection rates and improve hepatic, renal and immune function (Savino, 2018). Coconut and palm

kernel oils are considered to be a good source of MCT (Marten *et al*., 2006; FAO/WHO, 2001).

Cheese whey is the major by-product in the manufacture of cheese and its use is of industrial interest, considering its nutritional value, besides being a strong agent of environmental pollution (Monteiro *et al*., 2018; Alves *et al*., 2019; Guimarães *et al*., 2019; Trindade *et al*., 2019). Whey proteins have high biological value, possess functional and bioactive properties, and may be a protein source for enteral formulas, due to the evidence that they increase the synthesis of protein and can promote better tolerance of enteral nutrition (Abrahão, 2012; Silva *et al*., 2014; Guimarães *et al*., 2019). Recently, there has been interest in using whey proteins and hydrocolloids in food beverages, because this interaction may play an important role in solutions stability (Ahmadi *et al*., 2018; Guimarães *et al*., 2018a).

Inulin is one of the most used sources of dietary fiber in enteral formulas since it does not significantly interfere in the physical characteristics and stability compared to other fibers. It also provide a potential benefit in the prevention of diarrhea associated with enteral nutrition and may improve gastrointestinal health (Brown *et al*., 2015; Aydinol *et al*., 2018; Guimarães *et al*., 2018b; Moghadam *et al*., 2019; Silva *et al*., 2019) being able to modulate the intestinal microbiota and by its prebiotic effects (Generoso *et al*., 2016; Jakobsen *et al*., 2017; Teimouri *et al*., 2018).

The improvement of the nutritional quality of enteral formulas is a great opportunity for both patients and food industries. So far, the influence of variable ingredients addition in enteral formulas is not clarified in literature. Therefore, this study emphasizes the understanding of the influence of carbohydrates, lipids and proteins modification on stability and nutritional properties of enteral formulas.

This study aimed to evaluate the impact of inulin, MCT and whey proteins on emulsion stability and protein digestibility of enteral formulas. Thus, we used a centroid multiplex to test the influence of each component over the formulas.

2 MATERIAL AND METHODS

2.1 Experimental design and formulas development

For enteral formulas development, a *simplex-centroid* design was used. To assess the impact of raw material, three independent variables: inulin (I; X_1), medium-chain triglycerides (MCT; X_2), and whey protein isolate (WPI; X_3) were used in different ratios (Table 1). The proportion for each variable ingredient was expressed as a fraction of the total mixture, and the sum was always equal to 4%.

Table 1. Variable ingredients (%) used in the formulations according to the experimental design

Variable ingredients	C	I	M	W	IM	IW	MW	IMW
Inulin (X_1)[a]	0.00	4.00	0.00	0.00	2.00	2.00	0.00	1.33
MCT (X_2)[b]	0.00	0.00	4.00	0.00	2.00	0.00	2.00	1.33
WPI (X_3)[c]	0.00	0.00	0.00	4.00	0.00	2.00	2.00	1.33
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

WPI: Whey protein isolate; MCT: medium-chain triglycerides (palm kernel oil). C: control; I: inulin formula; M: MCT formula; W: WPI formula; IM: inulin and MCT formula; IW: inulin and WPI formula; MW: MCT and WPI formula; IMW: inulin, MCT and WPI formula.

[a] Orafti GR, Beneo, Oreye, Belgium
[b] Na palma, Belo Horizonte, Brazil
[c] Lacprodan 9224, Arla Foods, Denmark

Each formula was produced in duplicate, and the results are reported as the average of the batches. Preliminary tests were carried out for the establishment of enteral formulas composition according to the Brazilian Regulation for enteral nutrition formulas, which establishes levels according to the Total Energy Content (TEC) of the product (Anvisa, 2015).

The proteins content should be between 10 and 20%, lipids between 15 and 35%, whereas carbohydrates should be from 45 to 75%. The macronutrient levels were determined in order to remain within the limits established by legislation, even with the addition of different amounts of the variable ingredients. In addition to macronutrients, dietary fiber (up to 2.0 g/100 kcal) may be added, and the formula containing the highest content (I), this parameter achieved 0.9 g/100 kcal (Anvisa, 2015).

Then, the final formulations (100 g) were homogenized and stored in sealed aluminum foil bags (195 × 125 mm) under refrigeration (4°C).

2.2 Physicochemical and rheological analysis

The physicochemical analyses were carried out according to the Association of Official Analytical Chemists (AOAC, 2012). For moisture content, the samples were heated at 102°C until constant weight and ashes were determined by incineration at 550°C. For lipids, the analysis was carried out by Roese-Gottlieb method and proteins were determined by micro-Kjeldahl, using 6.38 as the conversion factor. The carbohydrates were estimated by difference in centesimal composition of enteral formulas.

The energy density was calculated by Atwater's factors. For pH measurement, the formulas were suspended in purified water (1.0 kcal/mL). Apparent viscosity was determined in Brookfield model DV-III viscometer (Middleboro, USA), spindle CP40 at 50 rpm, at 25^{o}C. All physicochemical measurements were done in triplicate, from two independent batches.

2.3 Emulsion stability

The emulsion stability was evaluated by droplet size and zeta potential. The droplet size distribution was determined by laser diffraction (LS 13 320, Beckman Coulter Life

Sciences, Indianapolis, IN, USA). Purified water (25°C), was used as a carrier agent for particle dispersion. The average diameter of the particles was determined based on the diameter of the same-volume sphere, De Brouckere diameter $D_{4,3}$ presented in Equation 1 (Mugele & Evans, 1951):

$$D_{4,3} = \frac{\sum_{i=1}^{n} n.d_i^4}{\sum_{i=1}^{n} n.d_i^3} \quad (1)$$

Where d_i, the diameter of the particles; n, number of particles.

Zeta potential was performed on a Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK). The samples were diluted in ultrapure water (10 μL/50 mL, 25°C). All measurements were taken in triplicate, from two independent batches.

2.4 In vitro digestion

Enteral formulas were digested *in vitro* according to the modified version of the standardized digestion method described by Minekus *et al*., 2014. For the simulated digestion phases, three stock solutions were prepared to simulate salivary (SSS), gastric (SGS) and intestinal fluids (SIS). To simulate the oral phase, the samples were diluted in approximately 3 mL of SSS until obtaining a homogeneous paste, followed by the addition of 5 mL of α-amylase solution (75 U mL^{-1}, Sigma-Aldrich A3176), at pH 7.0 for 2 min. For the next phase, the simulated gastric solution containing pepsin (2000 U mL^{-1}, Sigma-Aldrich, P7000) was added and pH adjusted to 3.0 (HCl 1 mol L^{-1}) and then incubated for 2 h. Finally, to simulate intestinal digestion, SIS containing pancreatin (100 U mL^{-1}, Merck 1.07130) and bile (10 mmol L^{-1}, Sigma-Aldrich B8631) was added, pH adjusted to 7.0 (NaOH 1 mol L^{-1}) and incubated for 2 h. All steps were performed at 37°C under constant gentle agitation at 250 rpm on a rotary shaking plate.

At the end of the procedure, the samples were centrifuged (5000 × *g* for 5 min) and the supernatant stored at -20°C for further analysis.

2.4.1 Protein digestibility assay – degree of hydrolisys

The degree of hydrolysis, before and after *in vitro* digestion, was measured by o-phthaldialdehyde (OPA) method as described previously by Morais *et al*., 2015.

2.4.2 Protein digestibility assay – peptide profile

For peptide profile, the samples were analyzed by Size-Exclusion High-Performance Liquid Chromatography (SE-HPLC). The analyses were done before and after digestion. It was performed in a PHEA column – poly (2-hydroxyethylaspartamide)-silica; 250 × 9.4 mm, 5 mm and 200 Å, detection at 230 nm. The samples were dissolved (2% w/v) in formic acid (0.05 $mol.L^{-1}$, pH 2.5) mobile phase and filtered through a 0.22 μm membrane. An aliquot (10 μL) was injected at room temperature, under isocratic conditions, at a 0.7 $mL\ min^{-1}$ flow rate, for 30 min (adapted from Morais *et al*., 2015). Ubiquitin (8,560 Da), insulin chain B oxidized (3,495 Da), N-Hippuryl-Histidyl-Leucine (429.47 Da), and alanine (89.09 Da) (Sigma-Aldrich, St. Louis, MO, USA) were used as peptide size standards.

The peptide profile was quantified and compared, dividing into four fractions: F1 (> 8.5 kD); F2 (3.5-8.5 kD); F3 (430 Da-3.5 kD); and F4 (< 430 Da), according to the retention time of standards. Peak area (mAU.min) was calculated using Empower 3 Chromatography Data Software (Waters, USA). An example of the chromatograms obtained is shown in Supplementary Material.

2.5 Statistical analisys

Data were compared by unifactorial analysis of variances (ANOVA One-Way) and *post hoc* Tukey's test with a critical value of 0.05. The effect of independent variables on the responses was evaluated by Response Surface Methodology (RSM), in agreement with the quadratic model, according to the Equation 2 (Karnopp *et al*., 2017):

$$\hat{Y} = b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (2)$$

Where $\hat{Y}$ was the predicted response; b_1, b_2, b_3, b_{12}, b_{13}, and b_{23} were the regression coefficients; and x_1, x_2, and x_3 were the codified factors.

All data were analyzed using Statistica 10.0 (Statsoft Inc. South America, Tulsa, OK, USA) and SPSS 15.0 (SPSS Inc., Chicago, IL, USA) software.

3 RESULTS AND DISCUSSION

3.1 Physicochemical and rheological analysis

All physicochemical analyses (Table 2) indicated differences between the formulas, except for moisture content (p = 0.57) and ash (p = 0.07). The pH and apparent viscosity varied little, even though samples were statistically different ($p < 0.001$), with a mean value of 5.98 (±0.06) for pH and viscosity of 8.65 (±0.35) cp.

Vieira *et al*. (2018) evaluated the nutritional quality of enteral formulas, commonly administrated to Brazilian patients, in their reconstituted form. The formulas herein developed, also diluted (in an adequate concentration to reach 1.0 kcal/mL), possess similar macronutrient composition, except for lipids content, which was less in the developed formulas (mean of 2.72 g/100 kcal) than in the formulas analyzed by those authors (4.3 g/100 kcal). The great variability of commercial formulas, in terms of nutrition content, is to attend different clinical indication.

Table 2. Physicochemical and rheological characterization of the formulas

Formulas	Moisture (%)	Ash (%)	Protein (%)	Lipids (%)	Carbohydrates (%)	Energetic Value (kcal/100 g)	pH	Apparent viscosity (cP)
C	5.17±0.13^{a}	3.66±0.18^{a}	16.04±0.39^{c}	10.52±0.40^{d}	64.61±0.77^{a}	417.24±2.32^{d}	5.98 ± 0.02^{a}	8.22 ± 0.24^{b}
I	5.21±0.30^{a}	3.26±0.45^{a}	16.90±0.83^{bc}	10.49±0.45^{d}	64.14±0.70^{ab}	409.35±2.83^{d}	6.04 ± 0.08^{ab}	8.47 ± 0.51^{ab}
M	4.93±0.36^{a}	3.58±0.14^{a}	16.11±0.09^{c}	14.07±0.18^{a}	61.32±0.36^{c}	436.30±1.84^{a}	5.99 ± 0.05^{ab}	8.77 ± 0.17^{a}
W	5.34±0.24^{a}	3.56±0.30^{a}	19.88±0.75^{a}	10.27±0.14^{d}	60.96±0.58^{c}	415.74±1.48^{d}	5.93 ± 0.05^{ab}	9.08 ± 0.28^{a}
IM	5.20±0.23^{a}	3.52±0.25^{a}	15.93±0.36^{c}	12.33±0.32^{b}	63.02±0.51^{b}	422.20±2.04^{bc}	5.93 ± 0.01^{b}	8.80 ± 0.15^{a}
IW	5.43±0.21^{a}	3.31±0.35^{a}	18.06±1.21^{b}	10.32±0.11^{d}	62.88±1.10^{b}	412.05±1.18^{d}	5.94 ± 0.05^{ab}	8.65 ± 0.18^{ab}
MW	5.09±0.10^{a}	3.69±0.08^{a}	18.07±1.11^{b}	12.19±0.33^{b}	60.96±1.26^{c}	425.80±1.50^{b}	5.97 ± 0.07^{a}	8.41 ± 0.06^{a}
IMW	5.17±0.23^{a}	3.46±0.19^{a}	17.02±0.41^{bc}	11.42±0.98^{c}	62.92±1.04^{b}	419.49±4.83^{cd}	6.05 ± 0.06^{ab}	8.85 ± 0.35^{b}
p-value*	0.57	0.07	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

*p-value by one-way ANOVA; Values are reported as mean ± standard deviation of three replicates.
^{a,b,c} Different letters in the same column represent significantly different values between samples. ($p < 0.05$)
C: control; I: 4% inulin formula; M: 4% MCT formula; W: 4% WPI formula; IM: 2% inulin and 2% MCT formula; IW: 2% inulin and 2% WPI formula; MW: 2% MCT and 2% WPI formula; IMW: 1.33% inulin, 1.33% MCT and 1.33% WPI formula.

The proposed mathematical model was not significant for apparent viscosity (p = 0.60) and pH (p = 0.87) and only a part of the data variance could be explained (R^2 = 0.406 for apparent viscosity and R^2 = 0.223 for pH), showing that the ingredients replacement did not affect these parameters.

3.2 Emulsion stability

The emulsion stability was evaluated on the diluted formulas, comparing then soon after dilution and after 24 h, under refrigerated storage (4 ^{o}C). Except for the mean droplet size after 24 h, the proposed mathematical model was not significant for the other analysis (initial droplet size and zeta potential), thus, these results will be discussed by mean comparison.

The initial droplet size (DS 0) showed that formulas M and IM, containing higher fat content (6.05 and 6.25 μm, respectively) presented a larger size than the others. Whereas those containing WPI resulted in smaller droplet size (W and IMW had 4.11 and 4.21 μm, respectively), possibly due to its emulsifying property, thus reducing fat droplets size (Fig. 1A) (Nishanthi *et al*., 2018).

Shimokawa *et al*., (2017), analyzing enteral formulas with different emulsifiers, observed mean droplet size between 194 and 250 nm, values over ten times smaller than those from this study (varying from 4.11 and 6.25 μm). Nevertheless, the emulsification method used consisted of vigorous stirring (3000 rpm for 10 min) and pressure homogenization (500 kgf/cm^2). Since the aim of this study was to reproduce home and hospital conditions, a domestic mixer was used, explaining the differences found.

For the zeta potential of the formulas, the initial mean value ranged from -45.28 and -39.75 mV. It is known that values above 30 mV, positively or negatively, are sufficient to ensure electrostatic stabilization (Castagnaro *et al*., 2013; Mohan *et al*., 2016). After 24 h of

refrigerated storage, the values varied from -42.25 to -35.33 mV, and as observed for droplet size, those containing higher fat content (M and MW) had lower stability, demonstrated by the differences between values obtained initially and after 24 hours (Fig 1B).

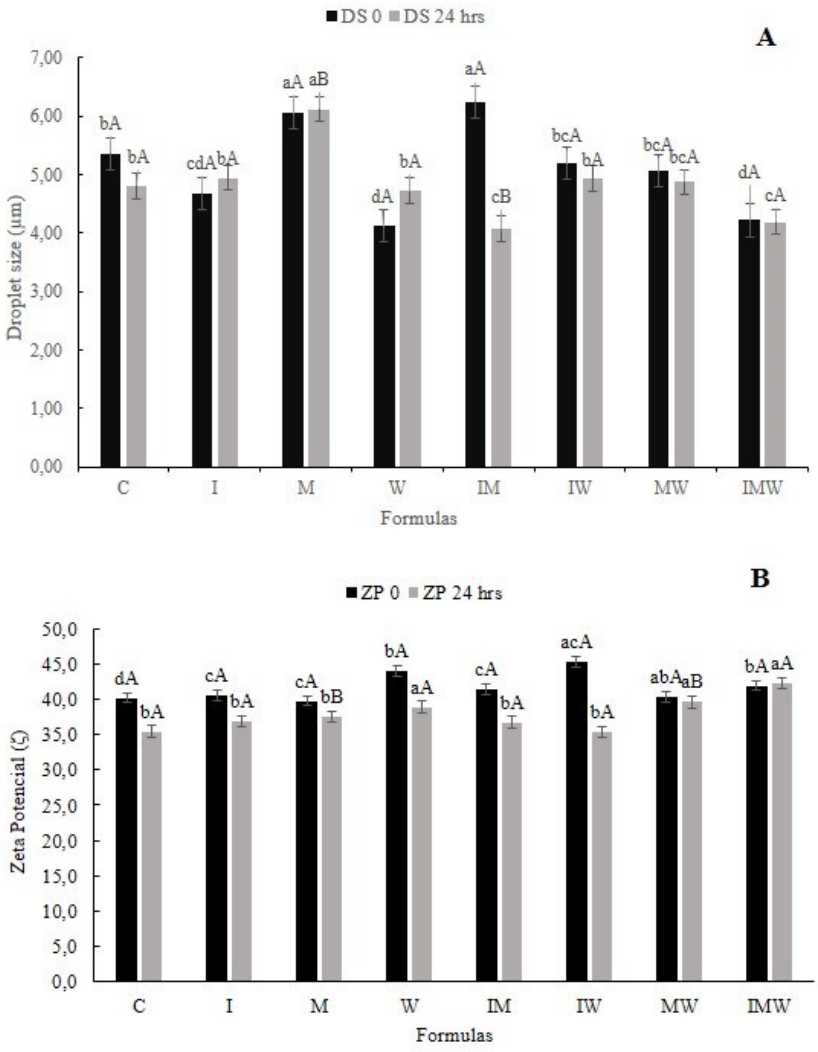

Figure 1. Mean droplet size (A) and zeta potential of the formulas (B), soon after its dilution in water and after 24 hours of refrigerated storage (4 °C).
[a,b,c] Different lowercase letters in the same column represent significantly different values between samples ($p < 0.05$). [A,B,C] Different uppercase letters in the same line represent significantly different values for the same sample ($p < 0.05$).

Some high zeta potential values were found for the formulas containing WPI, and these results could be related to WPI negative charges above its isoelectric point (4.6)

(Sriprablom *et al*., 2019). Since the formulas had a mean pH around 5.98, WPI influenced the zeta potential values, further reducing surface charges (Silvestre, 1999; González-Martínez *et al*., 2017).

A study performed by Wang *et al*., (2017) evaluated the influence of WPI and soybean lecithin on the stability of food emulsions. Similar results were observed, with zeta potential values around -45.0 mV. The authors attribute the negative and relatively high zeta potential values to a pH close to neutrality (5.1), above the whey proteins isoelectric point.

For droplet size after 24 h, at refrigerated storage (4°C), the model was significant ($p < 0.001$) and explained the experimental data ($R^2 = 0.9629$). This demonstrated that all ingredients influenced the mean droplet size. The interactions between inulin and MCT, as well as MCT and WPI, played a significant role in reducing the values, improving emulsion stability (Fig. 2).

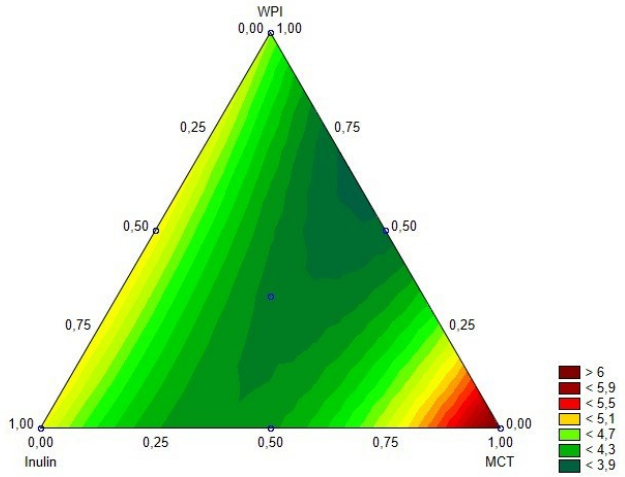

Figure 2. Response surface plot obtained by experimental model for droplet size after 24 hours of refrigerated storage (4 oC).

WPI: whey protein isolate; MCT: medium-chain triglycerides (palm kernel oil).

These data support that the interaction between factors was most beneficial for emulsion stability, considering that association of inulin and MCT or MCT and WPI reduced droplet size. In these cases, inulin and WPI acted as emulsifying agents, active substances that

can be absorbed in the oil droplet surface to produce an interfacial layer, providing electrostatic and steric stabilization (López-Castejón *et al*., 2019; Sriprablom *et al*., 2019).

3.3 Protein digestibility

The degree of hydrolysis (DH) of the intact formulas did not differ, presenting an average value of 5.17 ± 0.46%. However, after digestion, the values were between 15.27 and 19.01%. Peptide distribution, before and after digestion, demonstrated that in all formulas, larger peptides (> 10 kD) were predominant in the intact product, due to the mean molecular weight of the main whey proteins (β-lactoglobulin with 18.3 kD and α-lactoalbumin with 14.2 kD) (Jambrak *et al*., 2014). However, after digestion, smaller peptides predominated, especially below 0.4 kD, a fraction in which most of the di-tripeptides and free amino acids occur, which are the most available for intestinal absorption (Silvestre *et al*., 2011) (Table 3).

Table 3. Degree of hydrolysis of formulas (%) and peptide content (%) of the chromatographic fractions of the formulas, before and after in vitro digestion.

Formula	Degree of Hydrolysis (%)		F1 (> 8.5 kD)		F2 (3.5 kD - 8.5 kD)		F3 (0.5kD - 3.5 kD)		F4 (<0.5 kD)	
	Before	After	Before	After	Before	After	Before	After	Before	After
C	5.36 ± 0.86^{aB}	16.42 ± 1.18^{bcA}	97.42 ± 0.04^{aA}	0.38 ± 0.01^{dB}	0.03 ± 0.00^{cA}	20.66 ± 1.15^{aB}	0.34 ± 0.03^{aA}	26.07 ± 0.88^{bB}	2.21 ± 0.06^{eA}	52.89 ± 2.02^{bB}
I	4.97 ± 0.46^{aB}	15.27 ± 0.71^{cA}	83.77 ± 0.71^{cA}	0.32 ± 0.02^{dB}	0.17 ± 0.01^{cA}	15.34 ± 0.59^{bB}	0.00 ± 0.00^{cA}	42.23 ± 3.01^{aB}	16.06 ± 0.70^{aA}	42.11 ± 2.45^{cB}
M	4.92 ± 0.25^{aB}	18.66 ± 1.11^{aA}	86.95 ± 0.21^{cA}	0.38 ± 0.03^{dB}	0.14 ± 0.01^{cA}	16.60 ± 0.13^{bB}	0.00 ± 0.00^{cA}	21.04 ± 0.44^{bcB}	12.92 ± 0.22^{bA}	61.98 ± 0.27^{aB}
W	4.98 ± 0.40^{aB}	18.99 ± 1.41^{aA}	91.40 ± 0.13^{bcA}	0.72 ± 0.01^{abB}	3.38 ± 0.10^{aA}	16.31 ± 0.25^{bB}	0.06 ± 0.00^{bA}	20.55 ± 1.42^{bcB}	5.16 ± 0.03^{cA}	62.41 ± 1.68^{aB}
IM	5.41 ± 0.34^{aB}	16.73 ± 0.95^{cA}	97.69 ± 0.06^{aA}	0.52 ± 0.01^{cdB}	0.10 ± 0.00^{cA}	15.87 ± 0.00^{bB}	0.00 ± 0.00^{cA}	20.45 ± 0.15^{bcB}	2.21 ± 0.06^{eA}	63.17 ± 0.15^{aB}
IW	5.41 ± 0.29^{aB}	19.01 ± 1.49^{aA}	97.60 ± 0.21^{aA}	0.87 ± 0.06^{aB}	2.22 ± 0.19^{bA}	14.33 ± 0.16^{bB}	0.00 ± 0.00^{cA}	20.63 ± 0.76^{bcB}	0.18 ± 0.01^{fA}	64.17 ± 0.99^{aB}
MW	5.15 ± 0.21^{aB}	17.43 ± 0.90^{abA}	93.02 ± 3.85^{abA}	0.59 ± 0.07^{bcB}	0.30 ± 0.02^{cA}	13.58 ± 1.78^{bB}	0.00 ± 0.00^{cA}	18.22 ± 1.89^{cB}	3.88 ± 0.09^{dA}	67.60 ± 3.74^{aB}
IMW	$5,15 \pm 0.38^{aB}$	17.80 ± 1.17^{abA}	96.17 ± 0.10^{abA}	0.66 ± 0.03^{bcB}	3.44 ± 0.08^{aA}	14.51 ± 0.12^{bB}	0.00 ± 0.00^{cA}	19.69 ± 0.64^{cB}	0.38 ± 0.02^{fA}	65.14 ± 0.55^{aB}
p-value	0.085	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

*p-value by one-way ANOVA. Values are reported as mean ± standard deviation of three replicates.

^{a,b,c} Different lowercase letters in the same column represent significantly different values between samples ($p < 0.05$).

^{A,B,C} Different uppercase letters in the same line represent significantly different values for the same sample ($p < 0.05$)

C: control; I: 4% inulin formula; M: 4% MCT formula; W: 4% WPI formula; IM: 2% inulin and 2% MCT formula; IW: 2% inulin and 2% WPI formula; MW: 2% MCT and 2% WPI formula; IMW: 1.33% inulin, 1.33% MCT and 1.33% WPI formula.

To evaluate the influence of the three ingredients on the *in vitro* digestion of proteins, the significance of the proposed quadratic mathematical model for DH responses and F1, F3, and F4 fractions, after *in vitro* digestion, were evaluated (Fig. 3).

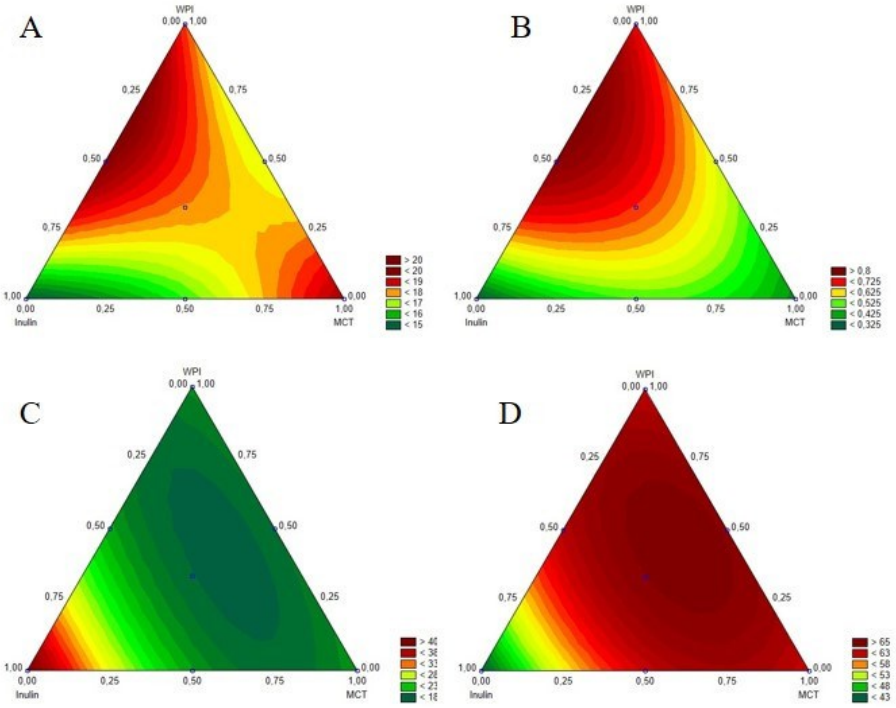

Figure 3. Response surface plot obtained by experimental model for Degree of Hydrolysis (A); F1 (B); F3 (C); F4 (D).

WPI: whey protein isolate; MCT: medium-chain triglycerides (palm kernel oil).

For DH, the values obtained by the mathematical model explained about 97% of the results. This demonstrates that all ingredients considerably contributed to increased DH, as well as the interaction between inulin and WPI. In contrast, MCT and WPI interaction had a negative influence. Among the pure components, inulin provided a smaller increase in hydrolysis degree, with the lowest coefficient, demonstrated by the response surface plot (Fig. 3A).

This influence may be due to the ability of inulin to gelation, which could interfere with the access of enzymes to immobilized proteins in gel network, thereby reducing the release of peptides and free amino acids (Luo *et al*., 2015; Simsek *et al*., 2017). The impact of food matrices was evaluated by Luo *et al*. (2015), who observed that, in solution, both whey and egg white proteins had faster digestion, with a higher degree of hydrolysis, compared to a gel matrix.

Digestibility was evaluated according to the distribution of peptides through analysis of fractions F1, F3, and F4, obtained after *in vitro* digestion. They are more representative of the hydrolysis process, and in intact formulas, for the same fraction, the differences were not pronounced.

Considering the F1 fraction (> 8.5 kD, large peptides), higher WPI contents (as a pure component or in interaction with inulin) provided higher residual content of large peptides, whereas inulin and MCT alone led to lower content (Fig 3B). However, for all formulas, the levels of large peptides remained below 1.0%, whereas for intact formula, the mean levels were 92.4% (Table 3). However, for the F3 fraction (0.4 kD – 3.5 kD), the interaction between MCT and WPI was not significant (p = 0.27), which confirms the minor importance of this combination for protein breakage (Fig 3C). Inulin as a pure component promoted higher peptide content, in the range of 0.4 to 3.5 kD (higher coefficient), and lower, in the range of less than 0.4 kD (F4), which demonstrates its influence on protein digestibility. According to the degree of hydrolysis data, this ingredient negatively affected the release of small peptides and free amino acids.

The use of MCT or WPI alone promoted higher coefficients for F4 (19.34 and 18.27, respectively) than for F3, demonstrating larger release of small peptides and free amino acids. Interactions among the factors were also significant, but with a lower effect on F3 and F4 (Fig 3C and 3D).

Fraction F4 represents the small peptides, in which most of the di-tripeptides are present, as well as the free amino acids. For this response, all factors and their interactions were significant, with the greatest influence given by pure MCT and WPI components, and least for inulin (Fig 3D).

Microstructure and processing of the food matrix are important factors that interfere with the digestion process and modify the release and absorption kinetics of nutrients, especially proteins (Rinaldi *et al*., 2014; Simsek *et al*., 2017). The impact of the structure of infant formulas on proteolysis, during *in vitro* digestion, was investigated by Bourlieu *et al*. (2015). They found that a minimally processed emulsion promoted a slower rate of protein digestion than in processed (homogenized and/or pasteurized) formulas, concluding that it is extremely relevant to evaluate how the structure of food influences digestion.

Simsek *et al*. (2017) evaluated three types of inulin (native, short-chain, and long-chain) on the release of peptides during *in vitro* digestion of low-fat caprines' milk kefir. They found no influence of the different types of inulin on the degree of digestion after duodenal phase, indicating complete hydrolysis of all milk proteins to smaller fragments, as detected by gel electrophoresis (SDS-PAGE), a different technique from that used in the present study. That technique was not able to produce quantitative differences for short peptides, containing less than five amino acid residues.

Our results are difficult to compare with data from the literature, considering that *in vitro* protocols vary widely. However, the method used (Minekus *et al*., 2014) is now considered the most reliable procedure to assess *in vitro* digestibility (Azzollini *et al*., 2018). Besides that, no other study evaluated the impact of different ingredients on digestibility of enteral formulas.

3.4 Multi-response optimization

The optimization for simultaneous responses was done numerically, according to the desirability function (Derringer & Suich 1980), using Design-Expert Software 11 (Stat-Ease, USA). The best combination of factors including minimum particle size after 24 h of refrigerated storage (4^{o}C); minimum F1 fraction after digestion (less large peptides); and maximum values of DH and F4 fraction after digestion. The numerical analysis suggested the formula with 0.70 g of inulin, 1.56 g of MCT, and 1.73 g of WPI, per 100 g, with an optimized product with a *d*-value = 0.769.

4 CONCLUSION

The ingredient replacement in enteral formulas significantly affected their nutritional composition, emulsion stability, and protein digestibility. Overall, the addition of MCT with inulin and WPI was beneficial to the emulsion stability of the product, while in those enriched with inulin, there was a reduced content of small peptides, related to a smaller digestibility of proteins. Thus, the desirability function was used to propose an optimized formulation containing 0.70 g of inulin, 1.56 g of MCT, and 1.73 g of WPI, per 100 g of formula, with an optimized product (*d*-value = 0.769). This optimized solution may be recommended for the production of enteral formulas because this has optimal emulsion stability and protein digestibility, which are essential characteristics for a product to be used by patients under special clinical conditions.

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Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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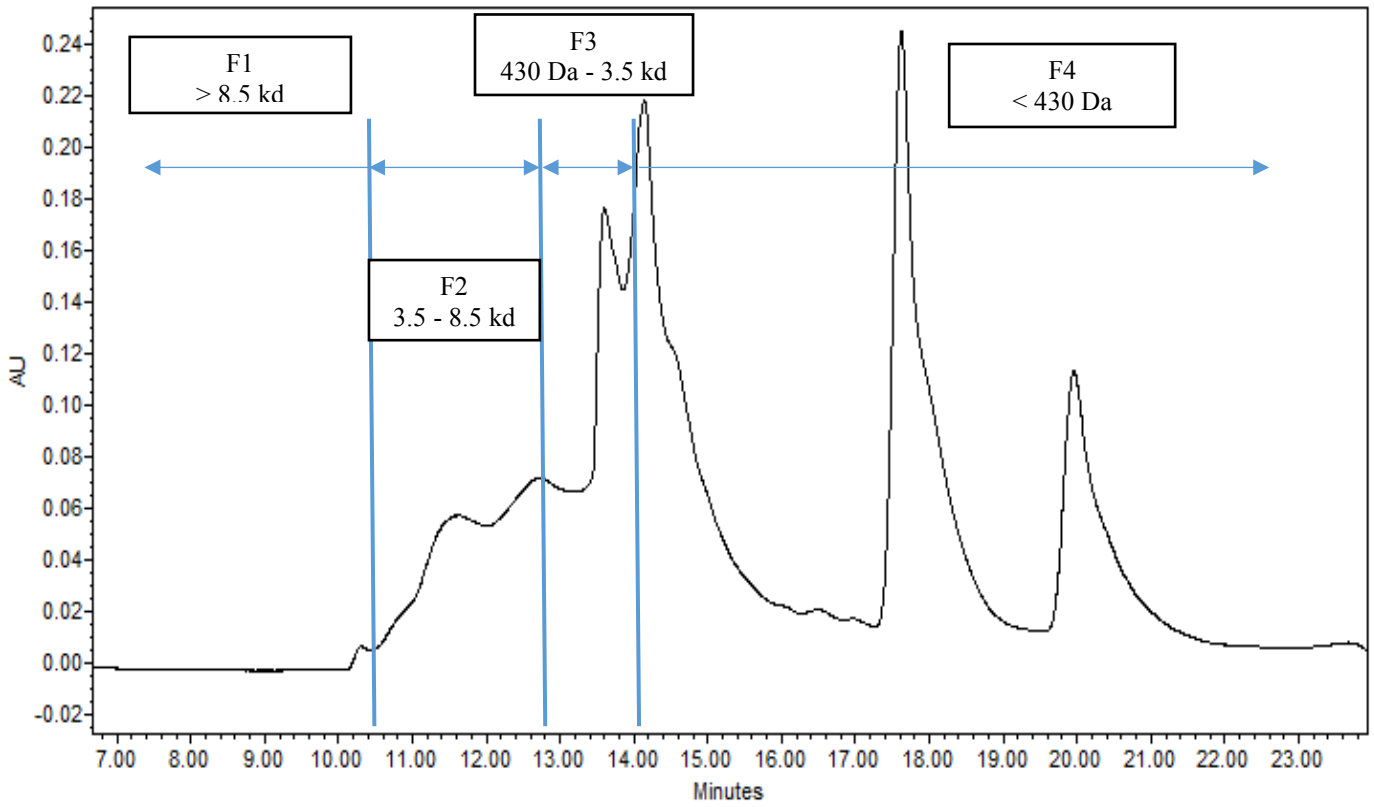
Supplementary Material

Example of size-exclusion chromatogram of enteral formula sample after simulated *in vitro* digestion.

Fractions and molecular weight ranges are indicated.



CHAPTER II

ENTERAL FORMULA AS VEHICLE FOR PROBIOTIC *Bifidobacterium longum* BL05: EVALUATION OF VIABILITY AND SURVIVAL UNDER *IN VITRO* SIMULATED GASTROINTESTINAL CONDITIONS

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ENTERAL FORMULA AS VEHICLE FOR PROBIOTIC *Bifidobacterium longum* BL05: EVALUATION OF VIABILITY AND SURVIVAL UNDER *IN VITRO* SIMULATED GASTROINTESTINAL CONDITIONS

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ABSTRACT

The aim of this study was to evaluate *Bifidobacterium longum* BL 05 viability during the storage and survival under *in vitro* simulated grastointestinal (TGI) conditions in powdered enteral nutritional formulas (ENF). Also, to assess the influence of different concentrations of inulin (X_1), medium-chain triglycerides (MCT) (X_2) and whey protein isolate (WPI) (X_3) as a protective effect on microbial cells. The ENF was storaged during 120 days at 4°C. The viability of probiotic microorganisms, water activity and pH were assessed until the end of the shelf life. Furthermore, the survival of probiotic under *in vitro* simulated gastrointestinal conditions was evaluated at the beginning of the storage. The formulas presented mean pH values of 6.00 and water activity of 0.534 after 120 days of storage. The populations count of *B. longum* BL 05 ranged from 9.05 to 9.79 log CFU g^{-1}, and the WPI conferred a protective factor. *B. longum* BL 05 demonstrated good survival when incorporated in enteral formulas and the presence of inulin protected from gastrointestinal conditions, achieving a minimum reduction of 2.03 log CFU g^{-1}, after 4 hours of simulated gastric and intestinal juices. An optimized solution was proposed with 1.78% inulin and 2.22% WPI, aiming the maximum probiotic viability and survival under gastrointestinal conditions by the desirability function (d = 0.875).

Keywords: probiotic, digestion, inulin, whey protein, enteral formulas.

1 INTRODUCTION

Enteral nutrition formulas (ENF) are industrialized products designed to provide complete or supplemental nutritional support, for patients unable to ingest conventional food. It can also be used as a source of specialized nutrition for patients with particular nutritional or physiological needs (Maka Taga *et al*. 2019). They are composed by intact or hydrolyzed protein and carbohydrates, lipids as vegetal oils or animal fat, besides vitamins, minerals and occasionally fibers. In the last decades, has been increasing the availability and variety of commercial enteral formulas, with different composition and indicated to several clinical conditions (Brasil 2015; Savino 2018).

Given their beneficial health potential, probiotics can improve the outcomes by the use of enteral formulas. Probiotics are defined as "live microorganisms which administered in adequate amounts confer a health benefit to the host" (Hill *et al*. 2014; Kumar and Salminen 2016). These benefits include modulation of the intestinal microbiota, protection against pathogens, improvement of the immune system and therapeutic applications in clinical conditions such as irritable bowel syndrome, ulcerative colitis and infectious diarrhea (Kumar and Salminen 2016). However, in order for their beneficial health effects to be achieved, probiotics must remain viable in adequate quantities in a food product during its manufacture and throughout shelf life (Dias *et al*. 2018).

Optionally, enteral formulas may contain fiber, such as inulin, which is an indigestible glucose polymer, and it has been widely used since it does not interfere in the formula physical characteristics and stability (Jakobsen *et al*. 2017). Besides that, it may exert a protective effect as prebiotic food ingredients, improving the survival and activity of probiotic bacteria during the storage of probiotic foods, as well as the passage through the gastrointestinal tract (GIT) (Rezaei *et al*. 2014). Other ingredients like whey protein concentrate may also present similar protective effects, increasing the maintenance during the shelf-life and the resistance against the pHs

changes and the enzymes secreted during passage through the GIT, allowing the probiotic bacteria to reach the intestine in a higher viable cell concentration (Akalin *et al*. 2007; Buriti *et al*. 2010). Furthermore, a lipid matrix probably protects bacterial cells from water and H^+ ions (Silva *et al*. 2017).

Considering the daily use of enteral formulas, its nutritional importance and the proposed health effects of probiotics, ENF could be a potential matrix for the incorporation of probiotics. Among the mostly used genera, the *Bifidobacterium* stands out for being one of the most widely used probiotic (Verruck *et al*. 2015). *Bifidobacterium longum* BL 05 was chosen because it is a commercial strain, which means that its safety and its beneficial properties has been previously demonstrated, besides being incorporated in other food matrices (Cruz *et al*. 2013; Lollo *et al*. 2013).

The aim of this study was to evaluate the viability of *B. longum* BL 05 during the storage of enteral nutrition formulas, its survival under *in vitro* gastrointestinal conditions and the influence of inulin, MCT and WPI on these parameters. Moreover, an optimized formula was proposed aiming maximum probiotic viability and survival.

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2 MATERIAL AND METHODS

2.1 Experimental design and enteral formulas preparation

Enteral nutrition formulas (ENF) (n = 9) were produced in two different batches according Brazilian Legislation (Anvisa, 2015). To assess the effect of ingredients [inulin (I; X_1), medium- chain triglycerides (MCT, X_2) and whey protein isolate (WPI; X_3)] a *simplex-centroid* design was used. Also, a control ENF was produced, without the ingredients I, MCT and WPI and an optimized formula, containing 0.70% of inulin, 1.56% of MTC and 1.74% of WPI, according to previously physicochemical analyzes (Souza *et al*. 2019). All samples were

produced with the freeze-dried probiotic culture of *Bifidobacterium longum* BL 05 (DuPont Danisco, Brabrand, Denmark) (Table 1).

For formulas preparing, all ingredients were weighted according to the dose indicated. The oily fraction was blended separately and then added to the powders, previously mixed in increasing order on a mortar. Finally, the mixture was sieved in a domestic strainer, in order to ensure homogenization. The freezed-dried cultures of *B. longum* BL 05 were added at the final step and manually mixed in sterile plastic bags. All formulas were stored under refrigeration (4 ^{o}C) in sealed aluminum laminated bags (195 x 125 mm), containing 100 g.

Table 1. Fixed and variable ingredients (%) used in the formulations according to the experimental design

Ingredients	C	I	M	W	IM	IW	MW	IMW	O
Fixed ingredients									
Maltodextrin[a]	60.90	56.90	56.90	56.90	56.90	56.90	56.90	56.90	56.90
WPI[b]	18.40	18.40	18.40	18.40	18.40	18.40	18.40	18.40	18.40
Cottonseed oil[c]	5.88	5.88	5.88	5.88	5.88	5.88	5.88	5.88	5.88
Canola oil[c]	3.08	3.08	3.08	3.08	3.08	3.08	3.08	3.08	3.08
Chia oil[d]	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84
Vitamins and minerals[e]	5.80	5.80	5.80	5.80	5.80	5.80	5.80	5.80	5.80
Soy lecithin[f]	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Xanthan gum[g]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
B. longum BL 05[h]	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Variable ingredients									
Inulin (X_1)[i]	0.00	4.00	0.00	0.00	2.00	2.00	0.00	1.33	0.70
MCT (X_2)[j]	0.00	0.00	4.00	0.00	2.00	0.00	2.00	1.33	1.56
WPI (X_3)[b]	0.00	0.00	0.00	4.00	0.00	2.00	2.00	1.33	1.74
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

WPI: Whey protein isolate; MCT: medium-chain triglycerides (palm kernel oil). C: control formula; I: inulin formula M: MCT formula; W: WPI formula; IM: inulin and MCT formula; IW: inulin and WPI formula; MW: MCT and WPI formula; IMW: inulin, MCT and WPI formula; O: optimized formula.

[a] Mor-rex 1910, Ingredion, Mogi Guaçu, Brazil
[b] Lacprodan 9224, Arla Foods, Denmark
[c] Bunge Brasil, Gaspar, Brazil
[d] Vila Alimentos, Vila Velha, Brazil
[e] SweetMix, Sorocaba, Brazil
[f] Planalto, São Leopoldo, Brazil
[g] Zibodan F200, Deosen, Piscataway, EUA
[h] Dupont Danisco
[i] Orafti GR, Beneo, Oreye, Belgium
[j] Na palma, Belo Horizonte, Brazil

2.2 Water activity and pH determination

The a_w was measured in Aqualab (Decagon Devices, USA) at 25 ^oC (Silva *et al*. 2017). For pH, the samples were diluted in purified water (1.0 kcal/mL), stirred in a vortex for 30 seconds and analyzed in a potentiometer (MS Tecnopon, Brazil). All measurements were taken soon after the preparation of the formulas and after 15, 30, 60, 90 and 120 days of refrigerated storage (4 ^oC), in triplicate.

2.3 Probiotic viability of *Bifidobacterium longum* BL 05 during storage

The probiotic viability was evaluated along the storage (0, 15, 30, 60, 90 and 120 days). The *B. longum* BL 05 count was done by decimal dilution in peptone water 0.1%, plating onto DeMan-Rogosa-Sharpe (MRS) agar (Acumedia, São Paulo, Brazil) supplemented with L-cysteine (0,1%) (Synth, Diadema, Brazil), and incubated at 37 ^oC for 72 hours, in anaerobic jars (Anaerobac, Probac, São Paulo, Brazil), according to Bernucci *et al*. (2017).

2.3.1 Growth potential

The growth potential (δ) of *Bifidobacterium longum* BL 05 was determined in each formula by calculating the difference between the microbial counts at the end of the shelf life (120 days) and at the beginning (log CFU g^{-1}) (Eq. 1). Formulations with a δ greater than 0.5 log_{10} were considered capable of supporting growth under the storage conditions studied (Munford *et al*. 2017).

$$Growth\ potential\ (\delta) = \log\left(N_1/N_0\right) \qquad (1)$$

where N_1 was the total viable count of bifidobacteria at the end of shelf life (120 days) and N_0 was the initial viable count bifidobacteria at the beginning

2.4 Probiotic survival under *in vitro* gastrointestinal conditions

The survival of *Bifidobacterium longum* BL 05 during *in vitro* simulated gastrointestinal conditions was evaluated on the free cells (freeze-dried DVS culture) and when incorporated on the nine formulas developed, by the protocol established by Krasaekoopt & Watcharapoka (2014), with modifications proposed by Bernucci *et al*. (2017). One gram of the free cells or the formula containing the probiotic bacteria (both with approximately 9 log CFU g^{-1}) was added to 10 mL of simulated gastric fluid (SGF). The SGF consisted of 0.08 mol L^{-1} HCl (Vetec, Rio de Janeiro, Brasil), 2 mg mL^{-1} NaCl (Synth, Diadema, Brazil), and 3 mg mL^{-1} pepsin (Sigma, St. Louis, USA), and the pH adjusted to 2.5 ± 0.1 with 1 mol L^{-1} HCl. This mixture was stirred in a vortex and incubated at 37 ^{o}C for 120 minutes. Then, it was centrifuged (2.792 x *g* for 10 minutes) and to the formed pellet was added 10 mL of simulated intestinal fluid (SIF). The SIF consisted of 0.05 mol mL^{-1} NaH_2PO_4 (Synth, Diadema, Brazil), 0.45 mg mL^{-1} bile salts (Sigma, St. Louis, USA) and 1.0 mg mL^{-1} pancreatin (Merck, Darmstadt, Germany), and the pH adjusted to 8.0 ± 0.1, with 1 mol L^{-1} NaOH. Samples were again incubated for 120 minutes at 37 ^{o}C. All solutions were sterilized by filtration through a 0.45 μm pore membrane (MF-Millipore, Billerica, MA, USA). Aliquots of 0.1 mL were withdrawn from the beginning (t = 0 min) and every 60 minutes, until the end (t = 240 min) of the experiments, for bacterial enumeration, as described at 2.3. All enumerations were taken in duplicate and the experiment was performed on the first day of storage.

2.4.1 Survival rate of bifidobacteria

In order to evaluate the bifidobacteria survival rate under simulated gastrointestinal conditions, Eq. 2 was used (Verruck *et al*. 2017).

$$Survival\ rate\ (\%) = \log \text{CFU}\ N_1 / \log \text{CFU}\ N_0 \times 100 \qquad (2)$$

where N_1 was the total viable count of bifidobacteria after exposure to simulated gastrointestinal conditions and N_0 was the initial viable count bifidobacteria before exposure to simulated gastrointestinal conditions.

2.5 Microbial quality of enteral formulas

Enumeration of total coliforms, total plate count, mold and yeast, coagulase positive staphylococci and the detection of *Salmonella* spp. were carried out according to the methods previously reported in the literature (Salfinger and Tortorello 2015). These analyses were performed at the first and at the last days of storage.

2.6 Statistical analisys

Data underwent unifactorial analysis of variances (ANOVA One-Way) followed by Tukey test for multiple comparisons, considering p = 0.05. To evaluate the effects of the variables on the responses, Response Surface Methodology (RSM) was employed, in agreement with quadratic model, according to Eq 3 (Karnopp *et al*. 2017):

$$\hat{Y} = b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{12} x_{12} + b_{13} x_{13} + b_{23} x_{23} \quad (3)$$

where $\hat{Y}$ is the predicted response and b_1, b_2, b_3, b_{12}, b_{13} and b_{23} were the regression coefficients and x_1, x_2 and x_3 were the codified factors.

All data were analysed using Statistica 10.0 (Statsoft Inc. South America, Tulsa, USA) and SPSS 15.0 (SPSS Inc., Chicago, IL, USA) softwares.

The optimization was obtained by using the Design-Expert Software (Version 11, Stat-Ease, Minneapolis, MN, USA). The desired goals for each independent variables were kept within the range and the responses were all maximized.

3 RESULTS AND DISCUSSION

3.1 Water activity and pH determination

All formulas showed a small but significant variation ($p < 0.05$) for pH among them, ranging from 5.90 to 6.12, but these values maintained similar during 120 days of storage (Table 2). These results demonstrate that the *B. longum* BL 05 was metabolically inactive during storage, and did not modified pH values.

The pH of commercial and home-made enteral formulas may range from 5.5 and 7.0 (Inoue *et al*. 2014; Sousa *et al*. 2014; Henriques *et al*. 2017). Whereas that pH rates lower than 4.6 may obstruct the feeding tubes, due to the coagulation of proteins, and if it is lower than 3.5, may interfere in gastric motility and contribute towards a slower gastric emptying (Henriques *et al*. 2017). Our results demonstrate that the formulas developed presented adequate pH values and compatible with enteral use. In addition, pH values between 5 and 8 are considered adequate for the growth and maintenance of bifidobacteria population (Gomes and Malcata 1999).

With regard to the water activity (a_w), it was observed a narrow range among analyzed samples, between 0.430 and 0.566, demonstrating that the variable ingredients and their concentrations did not influenced the a_w of the enteral formulas. Water activity is considered a critical factor to the stability of powered probiotic products, once these microorganisms present better survival rates with low water activity, although residual water is essential to preserve the structural stability of cell membranes (Vesterlund *et al*. 2012; Dias *et al*. 2018).

Table 2. pH and water activity during 120 days or refrigerated storage (4 °C)

Days of storage	Formulas																	
	FC		FI		FM		FW		FIM		FIW		FMW		FIMW		FO	
	pH	a_w	pH	a_w	pH	a_w	pH	a_w	pH	a_w	pH	a_w	pH	a_w	pH	a_w	pH	a_w
0	5.99^{cA} ± 0.01	0.450^{aA} ± 0.009	5.96^{deA} ± 0.01	0.445^{aA} ± 0.002	6.12^{aA} ± 0.01	0.432^{bA} ± 0.003	5.97^{eA} ± 0.01	0.430^{bA} ± 0.003	6.03^{bA} ± 0.01	0.447^{aA} ± 0.002	5.95^{deA} ± 0.01	0.438^{aA} ± 0.001	5.89^{fA} ± 0.00	0.442^{aA} ± 0.001	5.97^{cdA} ± 0.00	0.442^{aA} ± 0.008	5.99^{cdA} ± 0.01	0.461^{cA} ± 0.003
15	5.99^{bcdA} ± 0.02	0.456^{aA} ± 0.003	5.98^{cdA} ± 0.02	0.456^{aA} ± 0.001	6.09^{aA} ± 0.01	0.452^{aA} ± 0.001	5.96^{dA} ± 0.02	0.457^{aB} ± 0.002	6.02^{bA} ± 0.01	0.467^{bA} ± 0.001	5.99^{bcdA} ± 0.02	0.474^{bcB} ± 0.003	5.88^{eA} ± 0.01	0.467^{bB} ± 0.001	5.97^{cA} ± 0.02	0.465^{bA} ± 0.005	6.01^{bcA} ± 0.05	0.476^{cA} ± 0.001
30	6.01^{bcA} ± 0.01	0.482^{aB} ± 0.002	5.97^{dA} ± 0.02	0.467^{bA} ± 0.002	6.08^{aA} ± 0.02	0.466^{bB} ± 0.001	5.97^{dA} ± 0.01	0.465^{bB} ± 0.003	6.02^{bA} ± 0.02	0.474^{abA} ± 0.004	5.96^{dA} ± 0.02	0.488^{aC} ± 0.002	5.90^{eA} ± 0.02	0.485^{aB} ± 0.004	5.99^{bcdA} ± 0.01	0.483^{aA} ± 0.002	5.98^{cdA} ± 0.01	0.478^{aA} ± 0.002
60	5.99^{bcA} ± 0.01	0.521^{aC} ± 0.003	5.96^{cA} ± 0.02	0.545^{cB} ± 0.004	6.09^{aA} ± 0.01	0.532^{bC} ± 0.002	5.97^{bcA} ± 0.02	0.517^{aC} ± 0.003	6.01^{bA} ± 0.01	0.529^{bB} ± 0.004	5.97^{cA} ± 0.02	0.529^{bD} ± 0.003	5.92^{dA} ± 0.02	0.548^{cC} ± 0.001	5.98^{bcA} ± 0.02	0.550^{cB} ± 0.001	6.00^{bcA} ± 0.03	0.527^{abB} ± 0.002
90	5.98^{cdA} ± 0.01	0.536^{aC} ± 0.002	5.95^{dA} ± 0.01	0.525^{bB} ± 0.002	6.10^{aA} ± 0.01	0.531^{abC} ± 0.003	5.97^{cA} ± 0.01	0.566^{cD} ± 0.002	6.01^{bcA} ± 0.01	0.549^{dB} ± 0.003	5.97^{cA} ± 0.01	0.549^{dD} ± 0.003	5.89^{eA} ± 0.01	0.553^{dC} ± 0.002	5.99^{bcA} ± 0.01	0.545^{dB} ± 0.002	6.02^{bA} ± 0.01	0.566^{cB} ± 0.002
120	5.98^{cdA} ± 0.03	0.537^{abC} ± 0.013	5.96^{dA} ± 0.01	0.519^{bB} ± 0.011	6.11^{aA} ± 0.01	0.554^{bC} ± 0.012	5.97^{cdA} ± 0.02	0.528^{bCD} ± 0.012	6.03^{bA} ± 0.02	0.521^{abB} ± 0.001	5.98^{cdA} ± 0.01	0.525^{abD} ± 0.007	5.92^{eA} ± 0.01	0.554^{aC} ± 0.005	5.99^{cdA} ± 0.01	0.540^{abB} ± 0.009	6.01^{bcA} ± 0.01	0.545^{abB} ± 0.009

^{A,B,C} Different uppercase letters in the same column represent significantly different values between samples. ($p < 0.05$)

^{a,b,c} Different lowercase letters in the same line, for the same parameter, represent significantly different values between samples. ($p < 0.05$)

a_w: water activity; C: control formula; I: inulin formula M: MCT formula; W: WPI formula; IM: inulin and MCT formula; IW: inulin and WPI formula; MW: MCT and WPI formula; IMW: inulin, MCT and WPI formula; O: optimized formula.

The rate of food reactions and spoilage microorganisms activity is reduced with lower a_w, being retarded or even inhibited with a a_w below 0.3 (Dias *et al*. 2018; Ester *et al*. 2019). In our case, despite observing values above this critical point, no harmful or spoilage bacteria were found during storage. Our results are similar to the reported by Ester *et al*. (2019) for dried apple with *L. salivarius* microcapsules, in a range of 0.487 to 0.544, with a tendency to increase during storage time.

All formulas presented a slight increase, especially after 60 days of storage ($p < 0.05$), although they all remained under 0.6, which may contribute to the microbiological safety of the product, since almost none microorganism can grow under this condition (Vesterlund *et al*. 2012; Silva *et al*. 2017). The a_w increase was also observed by Tham *et al*. (2017) during 58 days of storage of commercially infant formulas.

Considering the ecology of the strain used in this study - anaerobic, the oxigen level within the package should be as low as possible. So, using sealed laminated bags, composed of layers of aluminum and polypropylene, which shows lower Oxygen and Water Vapor Transfer Rates, allowed the maintence of the populations counts along the storage (Cruz *et al*., 2007; Dutta and Dutta, 2016; Kumar *et al*., 2017).

The influence of the a_w on *Lactobacillus rhamnosus* GG viability was also evaluated by Vesterlund *et al*. (2012) in another dry matrix, ground flaxseed. Those authors observed that the lowest the a_w (0.11), the more extended the probiotic viability. However, the same product with a 0.43 a_w had a population decrease of approximately 3.7 log CFU g^{-1}, after 4 months of storage, at room temperature, which contrasts with the results of the present study. This difference is probably because of the complexity of the enteral formula matrix, which is composed by many nutrients that may protect the cells, and also due to the temperature of storage (Silva *et al*. 2017).

3.2 *Bifidobacterium longum* BL 05 viability in enteral formulas

The *Bifidobacterium longum* BL 05 counts (log CFU g^{-1}) during the 120 days of refrigerated storage (4 ^{o}C) are given in Table 3. It varied from 9.05 to 9.79 log CFU g^{-1}, with small, but significant differences ($p < 0.05$) among formulas and throughout storage. All formulas showed to be feasible as food matrices for the probiotic *B. longum* BL 05, since the average viability over the storage period was always above 9 log CFU g^{-1}.

Even though there is no consensus on the minimum effective concentration of probiotics to guarantee the proposed beneficial effects (Ouwehand, 2017), it is generally accepted that the products should have a minimum amount of 10^6 log CFU per mL/g, (Hill *et al*. 2014; Verruck *et al*. 2015; Dias *et al*. 2018; Mauro and Garcia 2019). Thus, the population counts observed in this study, ranging from 9.05 to 9.79 log CFU g^{-1}, during the entire storage period, are in agreement with the literature reported.

It can be observed that for almost all formulas there was an increase in population ($p < 0.05$) after 15 days of storage (Table 3), when compared to the initial counts. It was also reported by Dias *et al*. (2018) for microencapsulated *B. animalis* ssp. *lactis* BB12, and according to those authors, this increase cannot be attributed to cell multiplication, but it probably occurred as a consequence of the sub-lethally injured cells recovery during storage.

Although there were variations in the probiotic populations among the developed formulas, these differences were not microbiologically significant, since they were below 0.5 log CFU g^{-1} (Matias *et al*. 2016; Dos Santos *et al*. 2018).

Table 3. Probiotic counts (log CFU g^{-1}) and growth potential (δ) during 120 days or refrigerated storage (4 ^{o}C)

Probiotic counts (log CFU g^{-1})	Formulas								
	C	I	M	W	IM	IW	MW	IMW	O
T0	9.25^{Cc} ±0.02	9.56^{Aa}±0.03	9.59^{ABa}±0.03	9.05^{Bd}±0.03	9.06^{Cd}±0.04	9.30^{Bbc}±0.01	9.27^{BCc}±0.04	9.40^{Ab}±0.06	9.25^{Bc}±0.02
15d	9.73^{ABa}±0.05	9.64^{Aa}±0.07	9.65^{Aa}±0.07	9.44^{ABb}±0.01	9.51^{Bb}±0.01	9.75^{ABa}±0.06	9.38^{ABb}±0.05	9.75^{Aa}±0.01	9.73^{Aa}±0.06
30d	9.56^{Bab}±0.02	9.57^{Aab}±0.03	9.71^{Aa}±0.01	9.39^{ABb}±0.03	9.43^{BCb}±0.00	9.56^{ABab}±0.01	9.41^{ABb}±0.01	9.79^{Aa}±0.01	9.73^{Aab}±0.01
60d	9.73^{Aa}±0.00	9.70^{Ab}±0.02	9.73^{Aa} ±0.02	9.66^{ABb}±0.04	9.67^{Ab}±0.00	9.78^{Aa}±0.04	9.79^{Aa}±0.00	9.63^{Ab}±0.02	9.66^{ABb}±0.02
90d	9.47^{Bb}±0.03	9.38^{Bc}±0.01	9.56^{ABb}±0.05	9.83^{Aa}±0.02	9.46^{ABb}±0.03	9.76^{Aa}±0.01	9.68^{Aab}±0.01	9.54^{Abc}±0.02	9.59^{ABb}±0.17
120d	9.38^{Bb}±0.01	9.39^{Bb}±0.04	9.51^{Bb}±0.0 3	9.74^{Aa}±0.05	9.48^{ABb}±0.03	9.73^{Aab}±0.05	9.53^{Abb}±0.09	9.42^{Ab}±0.00	9.56^{Abb}±0.04
Growth potential (δ)	0.13	-0.17	-0.09	0.69	0.42	0.42	0.26	0.02	0.30

^{A,B,C} Different uppercase letters in the same column represent significantly different values between samples. ($p < 0.05$)

^{a,b,c} Different lowercase letters in the same line, represent significantly different values between samples. ($p < 0.05$)

C: control formula; I: inulin formula M: MCT formula; W: WPI formula; IM: inulin and MCT formula; IW: inulin and WPI formula; MW: MCT and WPI formula; IMW: inulin, MCT and WPI formula; O: optimized formula.

The proposed mathematical model was not significant for probiotic viability on days 0, 15, 30 and 60 of refrigerated storage. However, for the days 90 and 120 the model was significant and could explain 84% and 79% of the results, respectively. In addition, the lack of fit was not statistically significant (p=0.96 and p=0.37, respectively). A contour plot generated from this model (Fig. 1) confirmed that WPI was the most important variable ingredient to improve probiotic viability on day 90, and the interaction of WPI and inulin played a positive effect on day 120. Therefore, the calculated model was suitable for prediction on these storage periods.

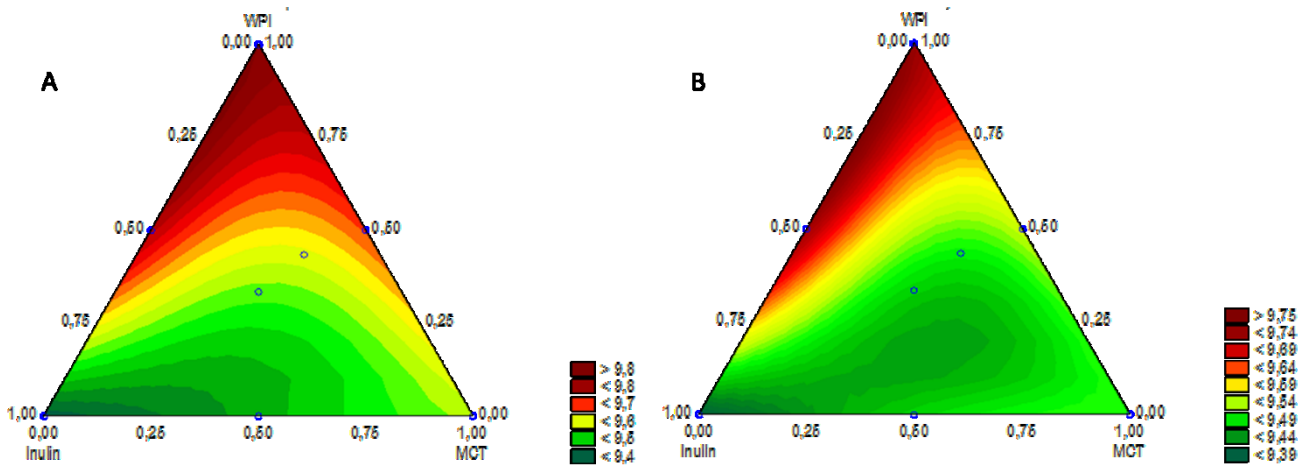

Fig 1. Response surface plot obtained by experimental model for probiotic counts (log CFU g^{-1}) after 90 days **(A)** and 120 days **(B)** of storage. WPI: Whey protein isolate; MCT: medium chain triglycerides (palm kernel oil).

The addition of whey proteins were also protective on the viability of *Lactobacillus acidophilus* La-5 incorporated in symbiotic guava mousses, when compared to milk fat and inulin, over 28 days of refrigerated storage. Similar to the results of the present study, inulin, by itself, did not improve the probiotic viability. (Buriti *et al*. 2010). Whey proteins added to yogurts have also been reported to improve the population counts of *Bifidobacterium* strains (McComas and Gilliland 2003; Akalin *et al*. 2007; Shori 2016). Those authors attributed this effect to the amino nitrogen content of whey proteins, as its buffering capacity. Yasmin *et al*

(2019) also reported that whey proteins, combined with pectin, were efficient on improving stability of microencapsulated *B. longum* BL 05 during long-term storage at 4 ^{o}C.

Furthermore, Matias *et al*. (2016) observed a decrease on *B. animalis* population on ice-creams supplemented with inulin, according to the results found in this study. The growth and viability of probiotic cultures in the presence of inulin varies with the degree of polymerization, being more efficient for short chains. Thus, the degree of polymerization, the environmental conditions, such as water activity, oxygen level and low storage temperature and the powder matrix, probably contributed to lack of effect of inulin on the viability of *B. longum* BL05 observed in this study (Pimentel 2012; Ozturkoglu-Budak *et al*. 2019).

In contrast, Rezazadeh-Bari *et al*., (2019) observed a decrease on *Lactobacillus acidophilus* La-5, after 21 days of storage of *Ayran* type yogurts, with the increasing concentration of whey proteins. Other authors (Souza *et al*. 2017) reported that inulin had a protective effect on the viability of probiotic *B. longum* BB12, over 35 days of refrigerated storage of margarines, compared to whey protein concentrate and caseinomacropeptide. This was not observed in this study, since enteral formulas supplemented with inulin had a slight decrease in probiotic populations after 120 days (-0.17 log CFU g^{-1}). These differences are mainly due to the variation in food matrices and the strains used (Ozturkoglu-Budak *et al*. 2019).

3.3 Survival of *Bifidobacterium longum* BL 05 in enteral formulas submitted to *in vitro* gastrointestinal conditions

Samples of *Bifidobacterium longum* BL 05, as free cells and incorporated on enteral formulas, were submitted to *in vitro* simulated gastrointestinal conditions. It aims to evaluate their survival to acidic pH of the stomach and to the bile salts of the intestine, which is essential to exert probiotic effects (Verruck *et al*. 2017).

Since the original counts of the freeze-dried commercial culture were above 10 log CFU g^{-1}, it was properly diluted in saline solution (NaCl 9 mg ml^{-1}) to achieve the same probiotic

concentration of enteral formulas. The survival rates and number of cells of *B. longum* BL 05 submitted to *in vitro* simulated gastrointestinal conditions is shown in Table 4. Over the incubation period (up to 4 hours), the average reductions in the probiotic populations varied from 2.03 to 3.39 log CFU g^{-1}. It was observed that the population counts at the end of the experiment, for all formulas and for free cells, were above 6 log CFU g^{-1} which is recommended for colonization in the intestine (Yasmin *et al*. 2019).

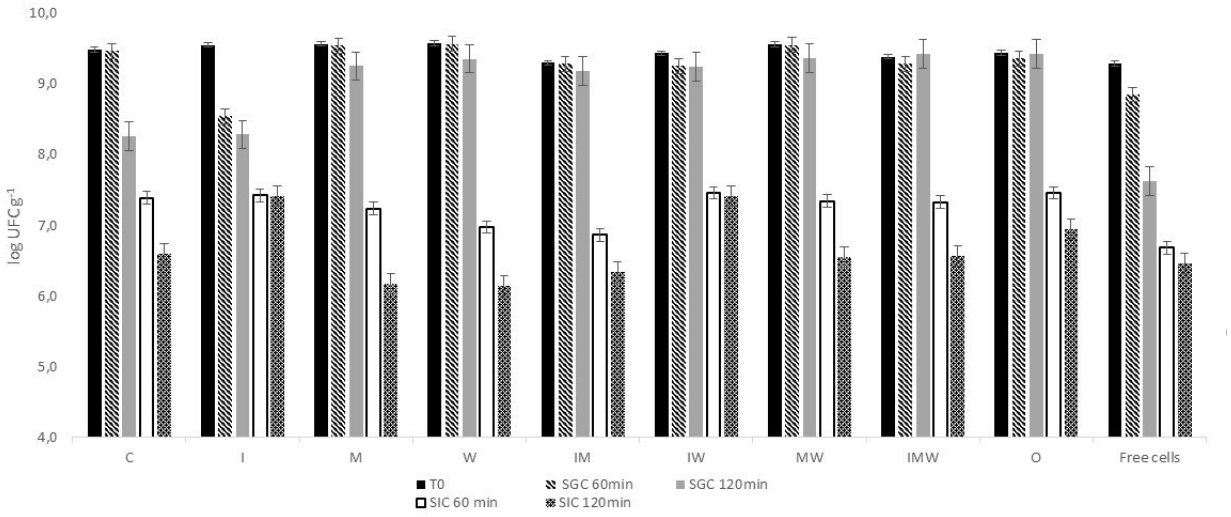

Fig 2. Survival of *B. longum* BL 05 (log CFU g^{-1}) submitted to *in vitro* simulated gastrointestinal at initial time (■) , after 60 minutes in simulated gastric conditions (▨); 120 minutes in simulated gastric conditions (▩); 60 minutes of *in vitro* simulated intestinal conditions (□) and 120 minutes of *in vitro* simulated intestinal conditions (▩ **).** ^{A,B,C} Different uppercase letters represent significantly different values between different incubation periods (T0; SGC 60min; SGC 120min; SIC60min; SIC120min) of the in vitro assay ($p < 0.05$).^{a,b,c} Different lowercase letters represent significantly differences between the populations between different formulas for each gastrointestinal phase ($p < 0.05$).

As seen in Figure 2, the initial probiotic count was equivalent for all formulas and free cells ($p > 0.05$). However, after the gastric phase, no significant reduction in probiotic population was observed when incorporated into all enteral formulas ($p > 0.05$), while for the free cells, there was a significant decrease after 2 hours of incubation in the simulated gastric conditions ($p < 0.05$), reinforcing the protective effect of the food matrix on acid pH resistance (Fig. 2).

The stomach conditions are very deleterious to some bacteria genres that cannot support the very low pH values and the presence of digestive enzymes, thus reducing the number of viable cells (Verruck *et al*. 2015; Rodrigues *et al*. 2019). Nevertheless, bacteria of the genera

Bifidobacterium are natural inhabitants of the human intestinal tract and are relatively stable during this conditions (Cruz *et al*. 2010).

The protective effect of the food matrix on bacterial counts under gastric conditions, when compared with the free cells, was also found by Verruck *et al*. (2015) in buffalo milk cheese. Also, a semisweet chocolate, with *Lactobacillus acidophilus* LA3 and *B. animalis* ssp. *lactis* BLC1, protected the probiotics to acid and bile salts (Silva *et al*. 2017) demonstrating the importance of choosing a suitable food matrix for the incorporation of probiotics (Dos Santos *et al*. 2018).

Rodrigues *et al*. (2019) also reported that the presence of a high concentration of total solids in food matrices might improve the ability of probiotic cells to support the gastric environment and physically preserve the cells against enzyme action.

Table 4. Number of surviving cells (log CFU g^{-1}) of *B. longum* BL 05 during sequential incubation (37 ^{o}C) in simulated gastric (SGC) and intestinal conditions (SIC)

Treatment	Inicial count	SGC		SIC		Survival Rate (%)	log reduction
		60 min	120 min	60 min	120 min		
C	$9.49^{Aa}\pm0.05$	$9.47^{Aa}\pm0.03$	$8.27^{Ab}\pm0.09$	$7.39^{Ba}\pm0.00$	$6.60^{Cbc}\pm0.09$	70	$2.89^{b}\pm0.04$
I	$9.56^{Aa}\pm0.04$	$8.55^{Ab}\pm0.02$	$8.29^{Ab}\pm0.15$	$7.42^{Ba}\pm0.06$	$7.42^{Ba}\pm0.05$	78	$2.14^{cd}\pm0.10$
M	$9.57^{Aa}\pm0.03$	$9.55^{Aa}\pm0.22$	$9.26^{Aa}\pm0.00$	$7.24^{Ba}\pm0.15$	$6.18^{Cd}\pm0.13$	65	$3.39^{a}\pm0.16$
W	$9.58^{Aa}\pm0.04$	$9.57^{Aa}\pm0.06$	$9.36^{Aa}\pm0.02$	$6.98^{Bb}\pm0.03$	$6.14^{Cd}\pm0.09$	64	$3.44^{a}\pm0.13$
IM	$9.30^{Aa}\pm0.11$	$9.29^{Aa}\pm0.15$	$9.19^{Aa}\pm0.09$	$6.86^{Bb}\pm0.11$	$6.33^{Bcd}\pm0.18$	68	$2.97^{b}\pm0.08$
IW	$9.44^{Aa}\pm0.07$	$9.26^{Aa}\pm0.04$	$9.25^{Aa}\pm0.02$	$7.46^{Ba}\pm0.07$	$7.41^{Ba}\pm0.06$	79	$2.03^{d}\pm0.01$
MW	$9.57^{Aa}\pm0.00$	$9.56^{Aa}\pm0.10$	$9.37^{Aa}\pm0.12$	$7.35^{Ba}\pm0.02$	$6.56^{Cc}\pm0.06$	69	$3.01^{b}\pm0.06$
IMW	$9.39^{Aa}\pm0.06$	$9.29^{Aa}\pm0.15$	$9.43^{Aa}\pm0.10$	$7.33^{Ba}\pm0.19$	$6.57^{Cbc}\pm0.08$	70	$2.82^{bc}\pm0.02$
O	$9.45^{Aa}\pm0.08$	$9.37^{Aa}\pm0.05$	$9.43^{Aa}\pm0.09$	$7.46^{Ba}\pm0.01$	$6.94^{Cb}\pm0.06$	74	$2.50^{c}\pm0.14$
Free cells	$9.29^{Aa}\pm0.17$	$8.85^{Ab}\pm0.10$	$7.63^{Bc}\pm0.00$	$6.68^{Cb}\pm0.04$	$6.46^{Ccd}\pm0.07$	69	$2.83^{bc}\pm0.10$

^{A,B,C} Different uppercase letters in the same line represent significantly different values between samples. ($p < 0.05$)

^{a,b,c} Different lowercase letters in the same column, represent significantly different values between samples. ($p < 0.05$)

C: control formula; I: inulin formula M: MCT formula; W: WPI formula; IM: inulin and MCT formula; IW: inulin and WPI formula; MW: MCT and WPI formula; IMW: inulin, MCT and WPI formula; O: optimized formula.

In order to evaluate the influence of the three variable ingredients (inulin, MCT and WPI), a quadratic model was generated for the variable responses, the population counts after each incubation time, that is SGC 60, SGC 120, SIC 60 and SIC 120. The contour plot generated for the survival of *B. longum* BL 05 in each step is presented in Fig. 3. The model was able to explain 89%, 95%, 76% and 87% of the results, respectively.

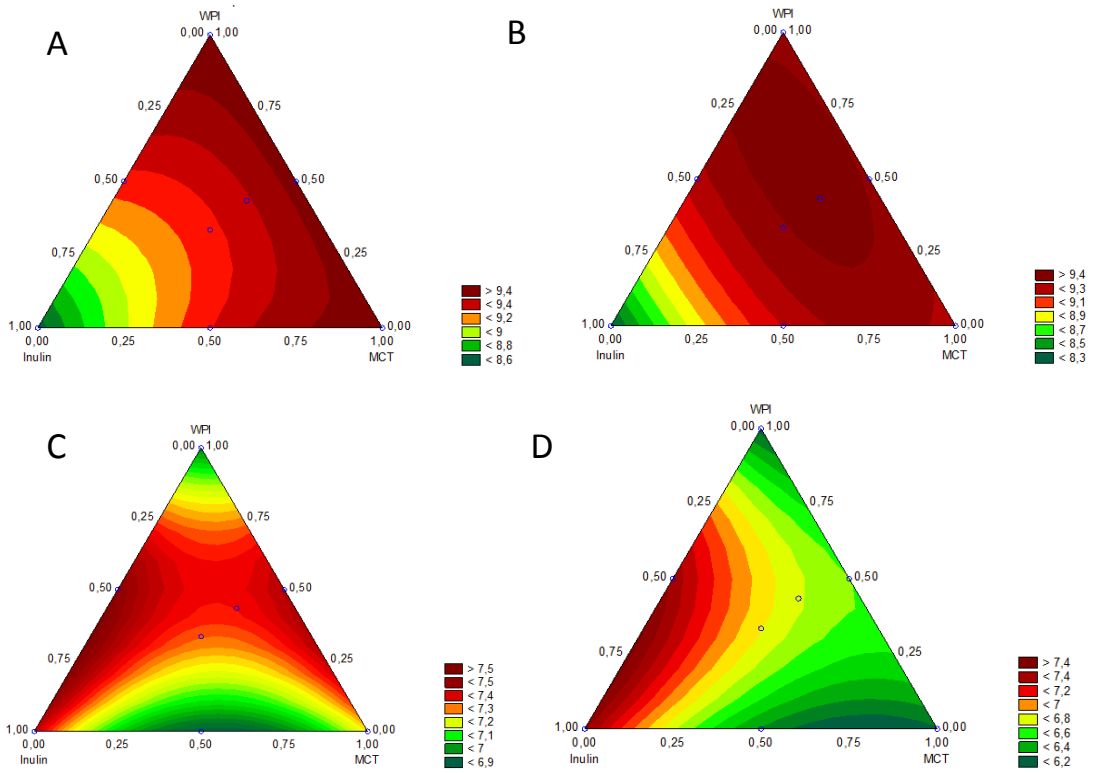

Fig 3. Response surface plot obtained by experimental model for probiotic survival (log CFU g^{-1}) after 60 minutes of *in vitro* simulated gastric conditions (**A**); 120 minutes of *in vitro* simulated gastric conditions (**B**); 60 minutes of *in vitro* simulated enteric conditions (**C**) and 120 minutes of *in vitro* simulated enteric conditions (**D**).
WPI: Whey protein isolate; MCT: medium chain triglycerides (palm kernel oil).

The model obtained revealed that the influence of the ingredients on probiotic survival varied from gastric to intestinal conditions. For the gastric phase (Fig 3.A, 3.B), whey proteins and MCT played a protective effect when compared to inulin. It may be explained by the buffer capacity and tolerance to low pH of the former and the ability of fat of reducing cells exposure to acid, which can protect bacteria against the harmful effects of the digestion environment (Ranadheera *et al*. 2012; Bernucci *et al*. 2017; Huang *et al*. 2017, Yasmin *et al*. 2019).

Notwithstanding, after 120 minutes of *in vitro* simulated intestinal conditions (Fig. 3.C), there is a tendency of inulin protective effect, even as its association with WPI and WPI/MCT. By the end of the simulated digestion process (Fig. 3.D), the effect of inulin on cells resistance is evidenced.

In guava symbiotic mousses, the substitution of milk fat by inulin was more favorable for the survival of *L. acidophilus* submitted to simulated gastrointestinal conditions (Buriti *et al*. 2010). This protection is probably associated with the resistance of inulin to hydrolysis by the gastrointestinal enzymes and its capacity to form gel when in solution, entrapping the probiotic cells inside the food matrix, thus avoiding the action of the enzymes on the cells (Buriti *et al*. 2010; Souza *et al*. 2017; Santos *et al*. 2019).

Similarly to what was observed in the present study, Souza *et al*. (2017) found that margarines supplemented with inulin presented lower reductions in the probiotic populations (*B. animalis* ssp. *lactis* BB12) after simulated gastrointestinal conditions (0.38 log CFU g^{-1}) when compared to WPI (3.6 log CFU g^{-1}) and caseinomacropeptide (1,84 log CFU g^{-1}).

Conversely, high percentage of fat in ice creams was shown to be more protective on different strains of *Lactobacillus* and *Bifidobacterium*, subjected to simulated digestion process, by reducing their exposure to bile salts and acid (Ranadheera *et al*. 2012).

The human gastrointestinal tract conditions are hostile for probiotic microorganisms, but the food matrix can protect the cells, avoiding great reductions in probiotic populations (Souza *et al*. 2017). Thereby, the food matrix, its compositions and physicochemical properties play an important factor on probiotic strains, as demonstrated in this study.

3.4 Microbiological quality of enteral formulas

The microbiological quality of the developed formulas were assessed in order to guarantee that other microorganisms would not interfere with the viability of probiotics along storage (Bennett *et al*. 2015). The results evidenced that the coliforms populations were below the detection limit of 3 MPN g^{-1} and *Salmonella*, coagulase positive staphylococci, mesophilic aerobics, molds and yeast were not detected in 25 g of enteral formulas, at the beginning and at the end of storage.

3.5 Multi-response optimization

The optimization for simultaneous responses was done numerically, according to the desirability function (Derringer and Suich 1980), to achieve the best combination of factors aiming maximum growth potential, maximum probiotic cell counts after 120 days of storage and maximum survival rate under simulated gastrointestinal conditions. The optimal solution predicted by the model suggested a formula containing 1.78 g of inulin, 2.22 g of WPI and without MCT per 100 g of formula, with an optimized product of desirability 0.875 (Fig 4).

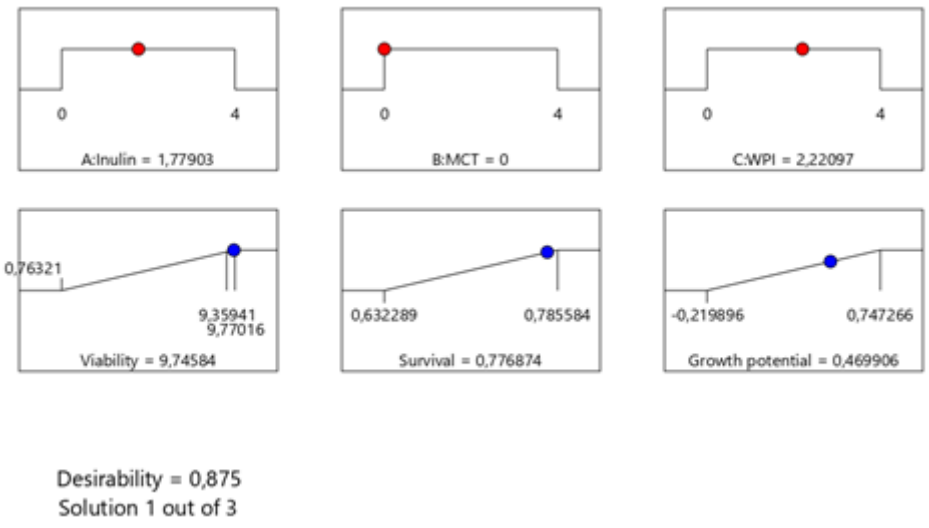

Fig 4. Simultaneous optimizations of the *B. longum* BL 05 viability, survival under *in vitro* gastrointestinal conditions and growth potential. MCT: medium-chain triglycerides; WPI: whey protein isolate

For this optimized solution the predicted values for enteral formulas were: 9.75 log CFU g^{-1} after 120 days of storage, 78% survival under *in vitro* gastrointestinal conditions and a growth potential of + 0.47. Thus, this solution may be recommended for enteral formulas production, providing the best conditions for viability and gastrointestinal resistance of *B. longum* BL 05.

4 CONCLUSION

Enteral formulas, containing especially inulin and WPI, are potential food matrices to improve viability and protect probiotic cells through the gastrointestinal tract passage. The proposed formulas promoted probiotic viabilities above 9.0 log CFU g^{-1} along 120 days of storage at 4 ^{o}C. *B. longum* BL 05 showed good survival under gastrointestinal conditions when incorporated to enteral formulas, and inulin played a protective effect. Optimization of the process demonstrated that 1.78 g of inulin and 2.22 g of WPI ensured the higher viability after 120 days of storage and adequate survival under gastrointestinal conditions.

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Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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FINAL CONSIDERATIONS

In a first step, the evaluation of the effect of the ingredients inulin, whey protein isolate, and medium-chain triglycerides demonstrated that they had a varied influence on the enteral nutrition formula composition and quality, especially in relation to emulsion stability and *in vitro* digestibility of proteins. The formulations developed met the requirements of composition foreseen in the Brazilian legislation and this matrix was able to be evaluated as vehicle for incorporation of probiotics.

Therefore, in a second phase, the effect of the same ingredients at the same concentrations was evaluated under the viability and resistance in simulated gastrointestinal tract conditions of the bifidobacteria incorporated to the formulas. The protective effect, especially of inulin and whey proteins on these parameters, was observed.

However, in order to assimilate the results obtained in both stages, it is suggested to use the numerical optimization tool in order to propose a final formulation, based on the answers obtained for each evaluated parameter. Thus, the desirability function was applied, as proposed by Derringer and Suich (1980), aiming: minimum particle size after 24 h of storage; minimum F1 fraction after digestion (less large peptides); maximum values of DH and F4 fraction after digestion, maximum probiotic cell counts after 120 days of storage and maximum survival rate under simulated gastrointestinal conditions.

The numerical analysis suggested a formula with 2.48% of inulin, 0.11% of MCT and 1.41% of WPI (*d*-value = 0.602).

This optimized solution may be recommended for the production of enteral formulas containing bifidobacteria, as a novel food matrix for probiotics, considering that it has been evaluated for its physicochemical and microbiological properties, which are essential characteristics for a product to be used by patients under special clinical conditions.