UNIVERSITY OF NAPLES "FEDERICO II"

DEPARTMENT OF AGRICULTURAL SCIENCES

AND

AN-NAJAH NATIONAL UNIVERSITY

FACULTY OF GRADUATE STUDIES

MASTER DEGREES IN

FOOD SCIENCE AND TECHNOLOGY
AND

NUTRITION AND FOOD TECHNOLOGY

Experimental thesis

EFFECT OF DAILY ADMINISTRATION OF MICROENCAPSULATED LIMOSILACTOBACILLUS REUTERI DSM 17938 ON THE INTESTINAL MICROBIOTA AND METABOLIC ALTERATION INDUCED BY HIGH FAT FRUCTOSE DIET IN ADULT RATS

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Dedication

I dedicate my dissertation work to my family, and many friends. A special feeling of gratitude to my loving parents, Nabil and Aida whose words of encouragement and push for tenacity ring in my ears. My brothers and sisters Jamal, Mohammad, Sara and Hala have never left my side and are very special.

I also dedicate this dissertation to my son and my husband's family who have supported me throughout the process. I will always appreciate all they have done.

I dedicate this work and give special thanks to my best professors Gianluigi Mauriallo, Arianna Mozolli, Francesca De Fillips and Mohammad Tamimi.

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الاقرار

أنا الموقع أدناه، مقدّم الرسالة التي تحمل العنوان:

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أقر بأن ما اشتملت عليه هذه الأطروحة إنما هو نتاج جهدي الخاص، باستثناء ما تمت الاشارة إليه حيثما ورد. وأن هذه الرسالة كاملة، أو اي جزء منها لم يقدم من قبل لنيل اي درجة او لقب علمي او بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: Jumana Nabeel Abuqurder **اسم الطالب:**

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Date: 26.7.2021 **التاريخ:**

List of Contents

List of Tables

List of Figures

List of Abbriviations

MetS	metabolic syndrome
HFF diet	high fat fructose
L.reuteri	limosilactobacillus reuteri
ALT	alanine transaminase
AST	aspartate amino transferase
ME	metabolizable energy

EFFECT OF DAILY ADMINISTRATION OF MICROENCAPSULATED LIMOSILACTOBACILLUS REUTERI DSM 17938 ON THE INTESTINAL MICROBIOTA AND METABOLIC ALTERATION INDUCED BY HIGH FAT FRUCTOSE DIET IN ADULT RATS

By
Jumana Abuqwider
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Abstract

Aims: To investigate the intestinal microbiota and metabolic alteration of 8-week oral supplementation with microencapsulated Lactobacillus reuteri DSM 17938 induced by high fat fructose diet in adult rats.

Materials and methods: In animal experiment, Male Wistar rats of 90 days were fed a control diet or a high fat - high fructose diet for 8 weeks, promoting signs of obesity and metabolic syndrome. Rats either fed a Control diet or HFF diet were divided into three groups. The first one, the control group (C) with the low-fat diet, the second, with the administration of the *L. reuteri* inside the microcapsule (CRM), while the last, were received simply *L. reuteri* (CR). In the same way the rats treated with a high fat - high fructose (HFF) diet were divided in three groups: one with the HFF (T), second group was administrated the *L. reuteri* inside the microcapsule (TRM), and the third group, was administrated by the HFF diet and the *L. reuteri* (TR). The research investigates the effect of supplementation on glucose homeostasis, body composition, energy balance, hepatic steatosis and fecal microbiota composition.

Results: Supplementation with microencapsulated *L. reuteri* DSM 17938 for 8 weeks did not affect significantly body composition and energy balance but affect significantly the plasma glucose and insulin resistance, liver steatosis, and microbiota composition. Rats who received the microencapsulated L. reuteri exhibited decreases in plasma glucose compared with control. In addition, microencapsulated L. reuteri supplementation significantly suppressed liver enzymes (AST, ALT) and hepatic steatosis, also increase the abundance of *Akkermansia muciniphila* which improves host metabolic parameters.

Conclusions: Intake of microencapsulated *L. reuteri* DSM 17938 for 8 weeks with diet induced obesity did not affect body composition and energy balance; however, it improved plasma glucose, insulin sensitivity, fatty liver

Aims of the thesis

The overall aim of this thesis is to contribute to the evidences regarding the effects of microencapsulated probiotic *Limosilactobacillus reuteri* DSM 17938 on the intestinal microbiota and metabolic alteration induced by high fat fructose diet. This general aim has been addressed through the implementation of novel technique uses to limit the considerable loss of viability of *L. reuteri* probiotic strain from gastrointestinal tract's harsh stress factors (acidic condition of stomach, digestive enzymes and bile salt) in a combination with a diet induce obesity and metabolic disturbances. In particular, the evaluation of 8 weeks oral administration of *L. reuteri*

microcapsule on glucose homeostasis, body composition, energy balance and gut microbiota composition along with high fat fructose diet in rat model highly resembles dietary habits of Western countries, i.e., consumption of a high-saturated fat diet combined with a high fructose intake.

Chapter One
Literature Review

1.1 Obesity and metabolic syndrome

The human environment, behaviors, and lifestyle have all dramatically changed in the last fifty years. Obesity and metabolic syndrome rates have risen as a result of these changes (Zimmet *et al*.,2005). In addition, they are major public health concerns due to their global prevalence and link to an elevated risk of chronic disease development (Andersen *et al*., 2016).

Obesity and weight gain are the result of a discrepancy between energy consumption and expenditure, and are a natural effect of over nutrition and a sedentary lifestyle (Misra and Khurana.,2008). The prevalence of obesity has increased over the past few decades. More than one-third of adults and 17% of children and adolescents in the United States are obese (Ogden *et al*., 2012). Also, it has been deemed the leading cause of preventable death (Jia *et al*., 2010) and has become a global economic and health burden (Misra & Khurana., 2008). Clinical manifestation of obesity tissue stress and dysfunction are often present as the parameters of metabolic syndrome (MetS), a condition characterized by a clustering of 3 or more of the following components (Jia *et al*., 2010): centrally distributed obesity, decreased high density lipoprotein (HDL) cholesterol, elevated triglyceride, high blood pressure, and hyperglycemia, connected with a threefold increase in type 2 diabetes and a twofold rise in cardiovascular disease, respectively (Zimmet *et al* ., 2005). In addition to these qualifying

parameters, obesity and MetS are associated with endothelial dysfunction, atherogenic dyslipidemia, insulin resistance, and chronic low-grade inflammation (Huang, 2009).

As obesity is the main key antecedent for metabolic syndrome that can be targeted in developing various therapies, several physical, psychological, pharmaceutical and dietary therapies have been proposed for the management of it. However, dietary practices have been demonstrated to be more effective without causing any negative health implications. Administration of probiotics as biotherapeutics is a relatively new field in the development of dietary approaches, and many people are curious about the science behind these health practices (Grover *et al*., 2012).

Probiotics are currently the focus of attention all over the world as potential biotherapeutics in the management of several inflammatory metabolic disorders, as they are now well recognized as powerful functional food and dietary ingredients with multiple health promoting functions along with their ability to fight specific diseases. However, because of the physiological effects attributed to probiotics are highly strain specific, strain selection may be critical in demonstrating their functional efficiency (Grover *et al*., 2012).

1.2 The gut microbiota

The term human microbiota characterizes the cooperative of microorganism’s occupation of our body, and the intestine being the main place in the body contain the largest number of microorganisms. At time,

the bacterial load in the human body is estimated to be about forty trillion (i.e., 3.8 x 10 13), mainly concentrated in the colon, and with a ratio against human cells of 1.3:1 (Sender *et al*., 2016). In the last years, more and more scientific papers have been published to describe the gut microbiota composition and their role in human and animal metabolism and physiology. Rapid increasing in number of papers happens with the coming of High-Throughput Next Generation Sequencing (HTNGS) technology. The advances of this technology have facilitated gut microbiome research and enabled the exploration of genetic and functional of intestinal bacteria population with reasonable costs and adequate productivity (Ji & Nielsen, 2015).

The microbiota of human adults has been extensively studied even though investigation into structural changes and compositional evolution from infants to the elderly has been only partially characterized. In healthy adults, 80% of the identified fecal microbiota can be classified into three dominant phyla: *Bacteroidetes*, *Firmicutes* and *Actinobacteria* (Harmsen *et al*., 2002). In general, terms the *Firmicutes* to *Bacteroidetes* proportion is considered to be an important formation the human intestine microbiota structure (Harmsen *et al*., 2002). On a more refined level, however, the fecal microbiota is a highly complex and diverse bacterial ecosystem. Within this ecosystem exists a hierarchy of dominant ($>10^9$ CFU/g) anaerobic bacteria, represented by the genera *Bacteroides, Eubacterium, Bifidobacterium, Peptostreptococcus, Ruminococcus, Clostridium* and *Propionibacterium*, and sub-dominant ($<10^9$ CFU/g) bacteria of the

Enterobacteriaceae family, especially *E. coli*, and the genera *Streptococcus, Enterococcus, Lactobacillus, Fusobacterium, Desulfovibrio* and *Methanobrevibacter (Mariat et al., 2009).* Gut microbiota has multiple functions and plays a crucial role in the human metabolism and physiology. First, gut microbiota forming the intestinal barrier, stimulate the continuous presence of gut microbiota, encourages intestinal epithelial cell reproduction, and produces mucus and nourishes mucosa by producing short chain fatty acids (SCFAs) (Burgervan Paassen *et al*., 2009). Gut microbiota is involved in the maturation of immune system by stimulating innate immune system in the early stage of life, which leads to the maturity of gut-associated lymphoid tissue (GALT), inspires the acquired immunity by stimulating local and systemic immune responses (Nell *et al*., 2010), intestinal synthesis and metabolism of certain nutrients, hormones and vitamins, and plays an important role in drug and poison removal (Rakoff Nahoum *et al*., 2004). Moreover, several studies in the last twenty years have described the role of gut microbiota in the production of important neurotransmitters and the microbiota-gut-brain axis concept was proposed (Cryan *et al*., 2015). First, studies in germ-free animals showed that the brain is affected in the absence of microbiota (Diaz Heijtz *et al*., 2011; Bercik *et al*., 2011). Second, animals given specific strains of bacteria had alterations in behavior (Bravo *et al*., 2011; Savignac *et al*., 2014), and human studies of such strains confirmed the potential translatability of such findings (Pinto-Sanchez *et al*., 2017; Tillisch *et al*., 2013). However, an unbalanced gut microbiota, which is often defined dysbiosis, can also cause

disease. Some common diseases in western countries such as obesity and type 2 diabetes have been contributed to shifts in the gut microbiota from state of eubiosis to state of dysbiosis, which is related to changes in composition and tasks of this bacteria, compared to healthy individuals. Dysbiosis may be occur through gain or loss of bacteria composition or alters in ratio of microorganisms. However, the main cause of dysbiosis of unhealthy individuals has been correlated with dietary habits (Wu *et al*., 2011; Menni *et al*., 2017). The new frontier of microbiota functionalities is constituted by the chemical interaction between microbiota and host cells to regulate gene expression. As mentioned before, microbiome genes quantity at least 100 times more than human genome (Qin *et al*., 2010). Thus, it is necessary to understand the metabolic interaction between the gut microbiota and the host genetic diversity in bacteria responsible for multiple function.

1.3 Cross talk between Gut microbiota and obesity and metabolic alteration

The link between gut microbiota, role in the host’s metabolic homeostasis and obesity has been widely established (Cani *et al*.,2009). Since type 2 diabetes and obesity are linked to low-grade inflammation and changes in gut microbiota composition, a bacterial component could have a role in the development of these disturbances induced by a high-fat diet. Several studies indicated that the bacterial substance was lipopolysaccharide (LPS), a component of gram-negative bacteria's cell wall (Delzenne *et al*.,2011).

One study showed that mice fed with high-fat diet exhibited enhanced levels of plasma LPS, a condition known as metabolic endotoxemia (Cani *et al*.,2007). Associations between circulating LPS level, consumption of a high fat diet and the presence of obesity and type 2 diabetes mellitus have been confirmed in humans too. Erridge and colleagues in 2007 found that a high-fat diet induces metabolic endotoxemia in healthy individuals.

Endotoxemia was linked to serum insulin and triglyceride levels, as well as an inverse relationship between endotoxemia and serum HDL cholesterol level, in people with type 2 diabetes and obesity. (Al-Attass *et al*.,2009). Furthermore, associations have been proposed between high-fat diet, metabolic endotoxemia and levels of inflammatory markers in mononuclear cells in Ghanim study in 2009 (Ghanim *et al*.,2009). Altogether, these findings support the theory that fat consumption and absorption, obesity, and the development of metabolic endotoxemia are all linked (Delzenne *et al*., 2011).

As mentioned before, some of the mechanisms that are involved in the development of metabolic endotoxemia seem to be related to the fat content of the diet (Delzenne *et al*.,2011). One investigator has shown that intraluminal fat increases intestinal LPS absorption through its incorporation into chylomicrons (Ghoshal *et al*.,2009). Accordingly, other investigator showed that the administration of lipase inhibitors reduces the severity of metabolic endotoxemia (Dixon *et al*.,2008). Cani and colleagues in 2008 found changes in the integrity of the intestinal barrier in both the

proximal and distal parts of the gut, potentially allowing LPS to enter the systemic circulation. Altered distribution and localization of two tight-junction proteins (ZO-1 and occludin) in the intestinal epithelium have been associated with an increased permeability of the intestinal wall in obese and diabetic rodents (Cani *et al*.,2008). Furthermore, glucagon-like peptide-2 (GLP-2), a gut peptide already known to be involved in the control of epithelial cell proliferation, was confirmed as a regulator of the expression and localization of tight-junction proteins and of the permeability of the intestinal wall in obese mice (Cani & Delzenne ,2009).

In order to identify the joining link between gut microbiota and metabolic pathologies, an intestinal permeability (IP) analysis is crucial. Over time, the study on IP has gained the interest of many research groups, highlighting the role that its increase could have in determining different metabolic disorders, both in animal and human models (Dumas *et al*.,2006; Wigg *et al*.,2001).

Even if the available literature does not uniformly agree on effective gut mucosal colonization (Markowiak & Slizewska ,2017), probiotics include microorganisms proven to exert positive effects on intestine and human health, such as *Lactobacillus* and *Bifidobacterium* genera (Fijan ,2014).

On the contrary, some intestinal bacteria are capable of activating inflammation pathways by interfering with the metabolism of lipids in adipocytes, macrophages, and vascular cells, inducing insulin resistance and the production of trimethylamine oxide. These facts represent the

pathogenetic environment to develop metabolic syndrome (Blaser & Falkow ,2009).

Finally, targeting microbiota may present new avenues for therapeutic interventions aimed at preventing or treating obesity and associated metabolic disorders. These strategies include dietary manipulation such as the use of prebiotics, probiotics or symbiotic, as well as transplantation of fecal microbial communities (Davis,2017).

Indeed, both genetic and physiological predispositions, as well as environmental factors, influence gut microbiome composition, and these factors work together to maintain gut homeostasis. As a consequence, maintaining a healthy gut microbiota composition is critical for both intestinal and distal appropriate host metabolic functioning (Yang and Kweon, 2016). (Figure 1) illustrates such interactions between the gut microbiota and the host metabolism.

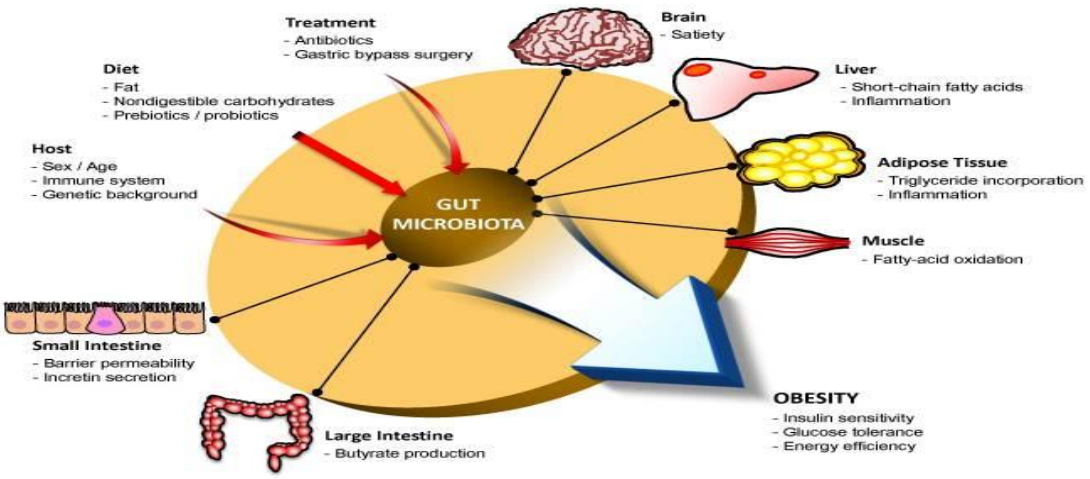

Fig 1: The gut microbiota and the host metabolism interact. External factors such as the host's environment, diet, and medical treatments can all have an impact on the gut microbiota. By affecting host insulin sensitivity or energy homeostasis, an imbalance of the gut microbiota can lead to severe metabolic diseases such as obesity and type 2 diabetes (Yang and Kweon, 2016).

1.4 Diet and gut microbiota

The main contributor to the diversity of the gut microbiota is diet (Maukonen & Saarela,2015; Rothe & Blaut,2013). It has been suggested that changes in the diet can account for 57% of the variations in microbiota compared to genetic variations in host that can only account for 12% (Brown *et al*.,2012). The effect of diet on microbiota composition is prominently observed as early as during breast and formula feeding as mentioned above. For example, level of *Bifidobacteria* is higher in breast-fed babies compared to formula fed babies (Pozo-Rubio *et al*.,2011; Roger et al.,2010). Moreover, probiotics and prebiotics are among the most dietary strategies established for controlling the composition and metabolic activity of gut microbiota (Baothman *et al*.,2016). Probiotics are non-pathogenic microorganisms used as food ingredients to benefit the hosts' health. Jones *et al.* (2012) investigated the effect of a bile salt-hydrolyase

Lactobacillus reuteri strain in hypercholesterolemic individuals. They found this strain can significantly lower the low-density lipoprotein cholesterol (LDL-C). Also, they proposed the role of nuclear receptor farnesoid X receptor (FXR) as transactional factor in reducing fat absorption from intestine. Furthermore, prebiotics have been shown to impact the host by specifically stimulating changes in the composition and/or activity of bacteria in the colon, and thus improving the hosts' health (Gibson & Roberfroid,1995). Lactulose, resistant starch and inulin are the most prebiotic compounds used by the food industry to modify the composition of gut microbiota to benefit human health. These have been shown to mostly target bifidobacteria and lactobacilli (Macfarlane *et al*., 2008). Prebiotics are carbohydrate-like compounds, such as lactulose and resistant starch, and have been used in the food industry to modify the composition of the microbiota species to benefit human health in recent years (Gibson *et al*.,2004). Inulin is one type of prebiotics. These prebiotics mostly target bifidobacteria and lactobacilli, which are two kinds of probiotics (Macfarlane *et al*.,2008). Recent research suggested that combining both prebiotics and probiotics, namely synbiotics can also fight obesity (De los Reyes-Gavilan *et al*.,2014).

The possibility that diet may be able to influence the gut microbiota has been discussed in the scientific community since the 1960s. Latest evidences have focused on using animal models and the analysis of intestinal microbiota and metagenomes to investigate the association between diet and the composition and function of the gut microbiome

(Hemarajata & Versalovic,2013). Human diets may have a direct impact on the microbiome, resulting in changes in metabolic reaction patterns in the intestinal lumen. Animals fed a high-fat, high-sugar Western diet showed rapid changes in intestinal microbial community structure, with increased numbers of members of the phylum Firmicutes and decreased abundance of members of the phylum Bacteroidetes, in experiments using germ-free mice transplanted with human fecal microbiota. However, after going back to a conventional chow diet for a week, microbial communities restored to their previous condition (Goodman *et al*.,2011). As a result, a high-fat, Western-style diet has been shown to influence the gut microbiota in a recent clinical trial in which healthy volunteers were fed with high-fat, Western-style diet for a month. Individuals who were fed a high-fat diet had higher plasma endotoxin levels than those who were fed an isocaloric regular diet, which may be a result of perturbations microbiome (Pendyala *et al*. 2012). However, it is still not known whether alterations of intestinal microbial communities represent causes or consequences of changes in human health status and different disease states (Hemarajata and Versalovic,2013).

The role of the diet in determining metabolic and inflammatory disorders, has modified in the last years, due to the huge scientific interest for gut microbiota (Federico *et al*., 2017). Pro or anti-inflammatory activity of some food would be only in part their intrinsic property, since in some cases, it can be linked to the formation of bacterial metabolites which, once absorbed in intestine, carry out a local and systemic action (Le Chatelier *et*

al.,2013). In this regard, dietary phospholipid phosphatidylcholine metabolism by bacterial enzymes causes the formation of trimethylamine (TMA) metabolized by hepatic Flavin monooxygenases to TMA Noxide, which is able to stimulate macrophage activity and induce atherosclerosis, a condition that could explain, at least in part, the higher cardiovascular risk in patients with both particular dietary habits and intestinal microbiota (Koeth *et al*.,2013). The diet also can be able to trigger metabolic and inflammatory alterations as happens in subjects who follow the Western diet, that is able to influence cytokine signaling, has a direct action on immune cells and increases toll-like receptors (TLR) expression and intestinal permeability (IP) (Pendyala *et al*.,2012). This harmful dietary habit causes a modification in intestinal microbial composition and large alterations associated with switching to the high-fat diet, including a decrease in *Bacteroidetes* and an increase in both *Firmicutes* and *Proteobacteria*, as happens in cases of high fat diet related dysbiosis (Hildebrandt *et al*.,2009).

Therefore, it is clear that any type of effect due to the diet in determining a large number of pathologies, cannot neglect the evaluation of possible roles carried out by gut microbiota. According to another point of view, the diet is able to modulate intestinal microbial composition, favoring the colonization of microbial species able to metabolize food, differently from health gut microbiota. In this way, a modification of interconnection between nutrients and health occurs. Moreover, the adage “we are what we

eat" could be changed into "we are what the gut microbiota transforms" (Federico *et al*., 2017).

1.5 Probiotic

Probiotics are defined as 'live microorganisms with Generally regarded as safe (GRAS) status, that when administered in adequate amounts, confer a health benefit on the host'. *Lactobacillus* and *Bifidobacterium* strains are the most often used probiotics (Hill *et al*.,2014).

The administration of probiotics as prospective biotherapies in the management of metabolic disorders, helps the body stay healthy in a variety of ways, colonizing resistance, generating acid, and short chain fatty acid (SCFA), controlling intestinal transit, restoring disturbed microbiota, boosting enterocyte turnover, and competitive exclusion of pathogens are all examples of common processes among examined probiotic strains (Hill *et al*., 2014). And in weight management with possible mechanisms of improved microbial balance, decreased food intake, decreased abdominal adiposity and increased mucosal integrity with decreased inflammatory tone (Grover *et al*.,2012).

Interestingly, probiotic must meet specific criteria in order to be deemed effective. These include the ability to resist in the gastrointestinal tract, a high tolerance to gastric acids, the lack of any antibiotic resistance genes that can be transmitted, and the ability to provide clear beneficial effects to the host (Montalban-Arques *et al*., 2015). For instance, supplementing with specific *Lactobacillus* and *Bifidobacterium* strains has been shown to (1)

improve epithelial and mucosal barrier functions, (2) inhibit the growth of pathogenic enteric bacteria and reduce the formation of pathogenic toxins, (3) mediate some of the negative effects associated with high-fat diets, (4) modulate the immune system, blood glucose levels and lipid profiles (Markowiak & Slizewska ,2017).

In a double blind, randomized placebo-controlled intervention trial was conducted on 87 subjects with high body mass index who were randomly assigned to receive *Lactobacillus gasseri* SBT 2055 (LG2055). The probiotic LG2055 was utilized as an auxiliary culture in yoghurt fermented with traditional yoghurt cultures, *Streptococcus thermophilus* and *L. delbrueckii ssp. bulgaricus*, in this investigation; yoghurt without LG2055 served as a placebo. According to the findings of this study, the probiotic strain significantly reduced abdominal adiposity, body weight, and other measurements, implying that it has a favorable effect on metabolic disorders (Kadooka *et al*.,2010). In another study, oral administration of *L. gasseri* BNR17 prevented increases in body weight and adipose tissue in diet-induced overweight rats (Kang *et al*.,2010).

Finally, probiotics appear to play a crucial role in metabolic syndrome and associated consequences, according to research conducted on animal experimental models, supplementation with *Lactobacillus rhamnosus* reduced visceral adiposity and diet-induced obesity in a mouse model of obesity, while also enhancing the integrity of the gastrointestinal microbial lining (Le Barz *et al*.,2019).

1.6 Probiotic microencapsulation

One of the most significant characteristics of a probiotic bacteria is that it must be viable in high concentration until it reaches the gut in order to achieve many of its health benefits on the host (Mattila-Sandholm *et al*.,2002). Furthermore, after the administration of a probiotic, there is, in many strains, a considerable loss of viability due to the gastrointestinal tract's harsh stress factors (e.g., acidic conditions of the stomach, digestive enzymes, and bile salts of the small intestine) (Charteris *et al*.,1998). The fact that the human digestive tract has such a diverse range of condition makes constructing a probiotic release system difficult, but it also allows for the creation of a highly customized system that targets the desired position (Cook *et al*.,2012).

The bacteria's survivability can be improved by microencapsulating them in a polymer matrix. It's critical that the microencapsulation matrix resists acid and that the preparation routine is delicate enough not to harm the entrapped cells. Furthermore, the polymer utilized must be non-cytotoxic and non-antimicrobial to ensure that neither the host nor the bacteria are damaged (Mandal *et al*.,2006). Microencapsulation in specialized ultrathin semipermeable polymer membranes has been successfully shown to protect live bacterial cells in oral and other delivery applications (Ainsley Reid *et al*.,2005).

1.7 Modification of the gut microbiota by the administration of probiotics

According to the actions exerted by human gut microbiota in humans (Ludovico Abenavoli *et al*.,2019) we can recognize three main mechanisms of action in obesity treatment by probiotics: antagonistic effects on pathogenic microorganism growth and competitive adherence to intestinal mucosa and epithelium (antimicrobial activity), increased intestinal mucus layer production and reduced intestinal permeability (barrier function), and modulation of the gastrointestinal immune system (immunomodulation) figure 1 (Thomas and Versalovic,2010). Altogether, these mechanisms can modulate gut microbiota composition and host metabolism, restoring a "lean gut microbiota" (Markowiak and Slizewska,2017).

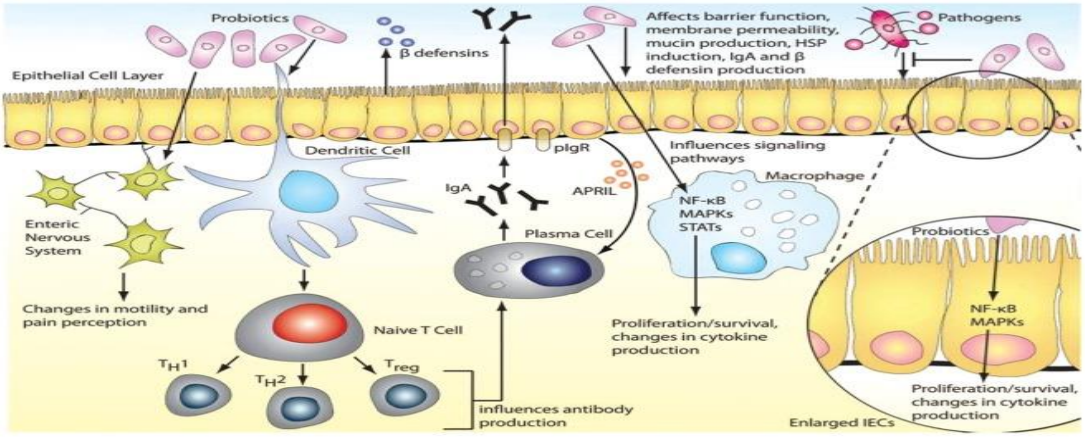

Fig 2: Probiotic mechanisms in the human gastrointestinal tract. Probiotics may manipulate intestinal microbial communities and suppress growth of pathogens by inducing the host's production of β-defensin and IgA. Probiotics may be able to fortify the intestinal barrier by maintaining tight junctions and inducing mucin production. Probiotic-mediated immunomodulation may occur through mediation of cytokine secretion through signaling pathways such as NFκB and MAPKs, which can also affect proliferation and differentiation of immune cells (such as T cells) or epithelial cells. Gut motility and nociception may be modulated through regulation of pain receptor expression and secretion of neurotransmitters (Thomas and Versalovic ,2010).

1.8 Animal experiment

Mention mice and rats to most people and images of unsanitary conditions and urban decay come to mind. But what is not always appreciated is the extraordinary impact that laboratory mice and rats have on biomedical research. They are often the preferred animal model for studies of human disease and the standard species of choice for pre-clinical trials. Mice and rats have long served as the preferred species for biomedical research animal models due to their anatomical, physiological, and genetic similarity to humans. Advantages of rodents are relatively small and require little space or resources to maintain, have short gestation times but relatively large numbers of offspring, and have fairly rapid development to adulthood and relatively short life spans. For example, mice have a gestation period of approximately 19-21 days; can be weaned at three to four weeks of age, and reach sexual maturity by five to six weeks of age, allowing large numbers of mice to be generated for studies fairly quickly. And also, they are abundant genetic resources (Bryda,2013). Additionally, laboratory rats and mice are abundant genetic resources, provide ideal animal models for biomedical research and comparative medicine studies because they have many similarities to humans in terms of anatomy and physiology. Likewise, rats, mice, and humans each have approximately 30,000 genes of which approximately 95% are shared by all three species (Sloane,2011; CDC,2012; Gibbs *et al*.,2004).

Rats are often the preferred rodent model for research where their larger size is an advantage, especially for facilitating surgical procedures and other types of testing. Many unique strains of rats have been generated that model the complex nature of human obesity, diabetes, and cardiovascular disease and therefore in this case, rats provide excellent animal models for the study of these diseases (Cowley *et al*.,2004; Kwitek-Black & Jacob,2001).

Chapter Two
Materials and Methods

2.1 Animals and Treatment

All experimental procedures involving animals were approved by "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University of Naples Federico II and authorized by Italian Health Minister (4448/2019-PR). This work complies with the animal ethics principles and regulations of the Italian Health Ministry. The authors ensured that all steps were taken to minimize the pain and suffering of the animals.

Male Wistar rats (Charles River, Calco, Lecco, Italy) of 90 days were caged singly in a temperature-controlled room (23 ± 1°C) with a 12 h light/dark cycle (06.30 – 18.30 h).

Rats were fed a control diet or a high fat - high fructose diet for 8 weeks, promoting signs of obesity and metabolic syndrome (Crescenzo R. *et al*., 2017). The composition of the two diets is shown in Table 1.

Rats either fed a Control diet or HFF diet were divided into three groups (each group constituted by 8 rats with the same mean body weight). The first one, the control group (C) with the low-fat diet, the second, with the administration of the 500 microliter *L. reuteri* inside the microcapsule (CRM), while the last, were received 100 microliters simply *L. reuteri* (CR).

In the same way the rats treated with a high fat - high fructose (HFF) diet were divided in three groups: one with the HFF (T), second group was administrated the *L. reuter*i inside the microcapsule (TRM), and the third group, was administrated by the HFF diet and the *L. reuteri* (TR) (Figure 3)

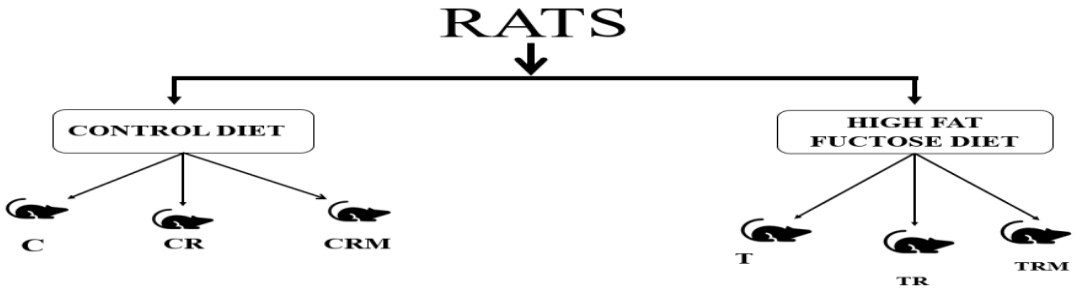

Figure 3: Experimental design

During the treatment, body weight and food intake were monitored daily. At the end of the experimental period, the rats were euthanized by decapitation, portal blood, systemic blood, cecum, liver, colon, pancreas, muscle, ileum as well as samples of WAT and IBAT, were collected and frozen at -80 ºC. Finally, carcasses were used for body composition determination.

Table 1: Composition of experimental diets.

Component	**Composition g/1000 g**	
	Low Fat	**High Fat- High Fructose**
Standard Cho**w**$^{\alpha}$**a**	395.3	231.5
Sunflower oil	19.3	19.3
Casein	59.7	133.3
Water	175.7	175.4
AIN-93 Mineral mix	11.4	11.4
AIN-93 Vitamin mix	3.2	3.2
Choline	0.7	0.7
Methionine	0.9	0.9
Cornstarch	333.8	0
Butter	0	129.8
Fructose	0	294.6
Energy content and composition		
ME content, kJ/g[b]	11.2	14.9

Lipids, J/100 J	10.5	39.3
Proteins, J/100 J	19.9	19.8
Complex carbohydrates, J/100 J	63.9	7.5
Simple sugars, J/100 J	5.7	33.4

[a]4RF21, Mucedola, Italy; [b]Estimated by computation using values (kJ/g) for energy content as follows: Protein 16.736, lipid 37.656, and carbohydrate 16.736. ME = metabolizable energy; AIN= American Institute of Nutrition.

2.2 GLUCOSE TOLERANCE TEST AND INSULIN DETERMINATION

Food was withdrawn at 08.00 a.m. After 6 hours, basal post-absorptive samples obtained from venous blood from a small tail clip were collected in EDTA-coated tubes and then glucose (2 g kg^{-1}) was injected intraperitoneally. Blood samples were collected after 20, 40, 60, 90, 120 and 150 min and placed in EDTA coated tubes. The blood samples were centrifuged at 1400 g for 8 min at 4°C. After centrifugation at 1400 g for 8 min at 4°C, plasma was isolated and stored at −20°C until used for determination of substrates and hormones. Plasma glucose concentration was measured by a colorimetric enzymatic method (Pokler Italia, Pontecagnano, Italy). Plasma insulin concentration will be determined using an ELISA kit (Mercodia AB, Uppsala, Sweden) in a single assay to avoid interassay variations. Basal postabsorptive values of plasma glucose and insulin were used to calculate the homeostatic model assessment (HOMA) index. The hepatic insulin resistance index will be calculated according to Abdul-Ghani et al. (2007). Briefly, after glucose injection, the increase in plasma glucose and insulin concentrations is proportional to the degree of hepatic insulin resistance *(Abdul-Ghani, 2007)*, the HOMA index was calculated using the formula: (Glucose (mg/dl) x Insulin (mU/ l))/405.

Hepatic insulin resistance index was calculated according to Abdul-Ghani (2007). Briefly, since the magnitude of the rise in plasma glucose and insulin concentrations immediately (0–30 min) following the glucose load is proportional to the magnitude of hepatic insulin resistance, we calculated the rise in plasma glucose and insulin concentrations during the period 0-30 minutes of the glucose load, by measuring the area under the curve (AUC) of plasma glucose and insulin. Then, hepatic insulin resistance index was calculated as: (glucose AUC 0-30) x (insulin AUC 0-30) *(Abdul-Ghani et al, 2007).*

2.3 Body composition and energy balance

Body composition was measured as previously described (Crescenzo R. *et al*, 2012). Briefly, the alimentary tract was cleaned of undigested food and the carcasses were then autoclaved. After dilution in distilled water and subsequent homogenization of the carcasses, duplicate samples of the homogenized carcass were analyzed for energy content by bomb calorimetry. Body lipid content was measured by the Folch extraction method (Folch J. *et al,* 1957). Body protein content was determined using a formula relating total energy value of the carcass, energy derived from fat, and energy derived from protein (Dullo AG, 1992) the caloric values for body fat and protein were taken as 39.2 and 23.5 kJ/g, respectively (Armsby, HP, 1917). Energy balance measurements were conducted by the comparative carcass technique over the experimental period, as detailed previously (Crescenzo R, *et al* 2013). Briefly, during the experimental

period, metabolizable energy (ME) intake was determined by subtracting the energy measured in faeces and urine from the gross energy intake, determined from daily food consumption and gross energy density of the diet. Body energy, fat and protein gain were calculated as the difference between the final and initial content of body energy, fat and protein. Energy expenditure was determined as the difference between ME intake and energy gain, and energetic efficiency was calculated as the percentage of body energy retained per ME intake. Lipid and protein partitioning was obtained by calculating the amount (expressed in %) of lipid or protein intake that was stored or oxidized.

2.4 Cultivation and microencapsulation of *Limosilactobacillus reuteri* DSM 17938

Limosilactobacillus reuteri DSM 17938 was isolated from Reuterin (Noos S.r.l.; BioGaia AB, Stockholm, Sweden) and cultured in MRS Broth (OXOID Ltd., Basingstoke, Hampshire, England) at 37 °C, checked for purity and maintained on MRS Agar (Oxoid). Free and microencapsulated cells of *L. reuteri* DSM 17938 were routinely cultured and counted on MRS Agar at 37 °C for 48 h in aerobic conditions. *Pseudomonas fragi* 25P used in reuterin production test, belonging to microorganism's collection of Department of Agriculture, University of Naples Federico II, was previously isolated from fresh meat sample (Ercolini, Russo, Nasi, Ferranti, & Villani, 2009). It was cultured in Tryptone Soya Broth (TSB, Oxoid) supplemented with 5 g/L Yeast Extract Powder (Oxoid) at 20 C.

Microencapsulation of bacterial cells was carried out by using the Encapsulator B-395 Pro equipped with a 120 mm nozzle and a syringe pump (BÜCHI Labortechnik, Flawil, Switzerland). A scheme of the Buchi encapsulator is reported in the (Figure 4) (De Prisco *et al*.,2014). In detail, the cells of a defined volume of *L. reuteri* DSM 17938 culture in the early stationary phase were harvested by centrifugation at 5200 g for 15 min. The cell pellet was washed once in an equal volume of a sterile quarter-strength Ringer solution (Ringer), harvested by centrifugation and finally suspended in an equal volume of a 12 g/L alginate (Sigma, Milan, Italy, product n. A2033) solution, previously degassed and sterilized, to reach a concentration of about 9.40 ± 0.10 Log. The syringe, used in the feeding system, was loaded with 50 ml of the alginate cell suspension and placed on the Encapsulator according to the instruction of supplier. The microencapsulation conditions used were: flow rate 2.91 ml/min, vibration frequency 1740 Hz, electrode voltage 950 mV. Alginate droplets containing bacterial cells were hardened in 200 ml of a 0.5 mol/L CaCl2 solution (in ratio 4:1 with alginate cell suspension) for about 20 min in stirring to obtain monodisperse cross-linked microcapsules. Suspension was left 30 min at room temperature for the sedimentation of microcapsules and then a volume of 150 ml of the upper phase was gently sucked and discarded to restore the initial cell concentration. Alginate microcapsules (MC) were routinely stored at 4 °C for further experiments.

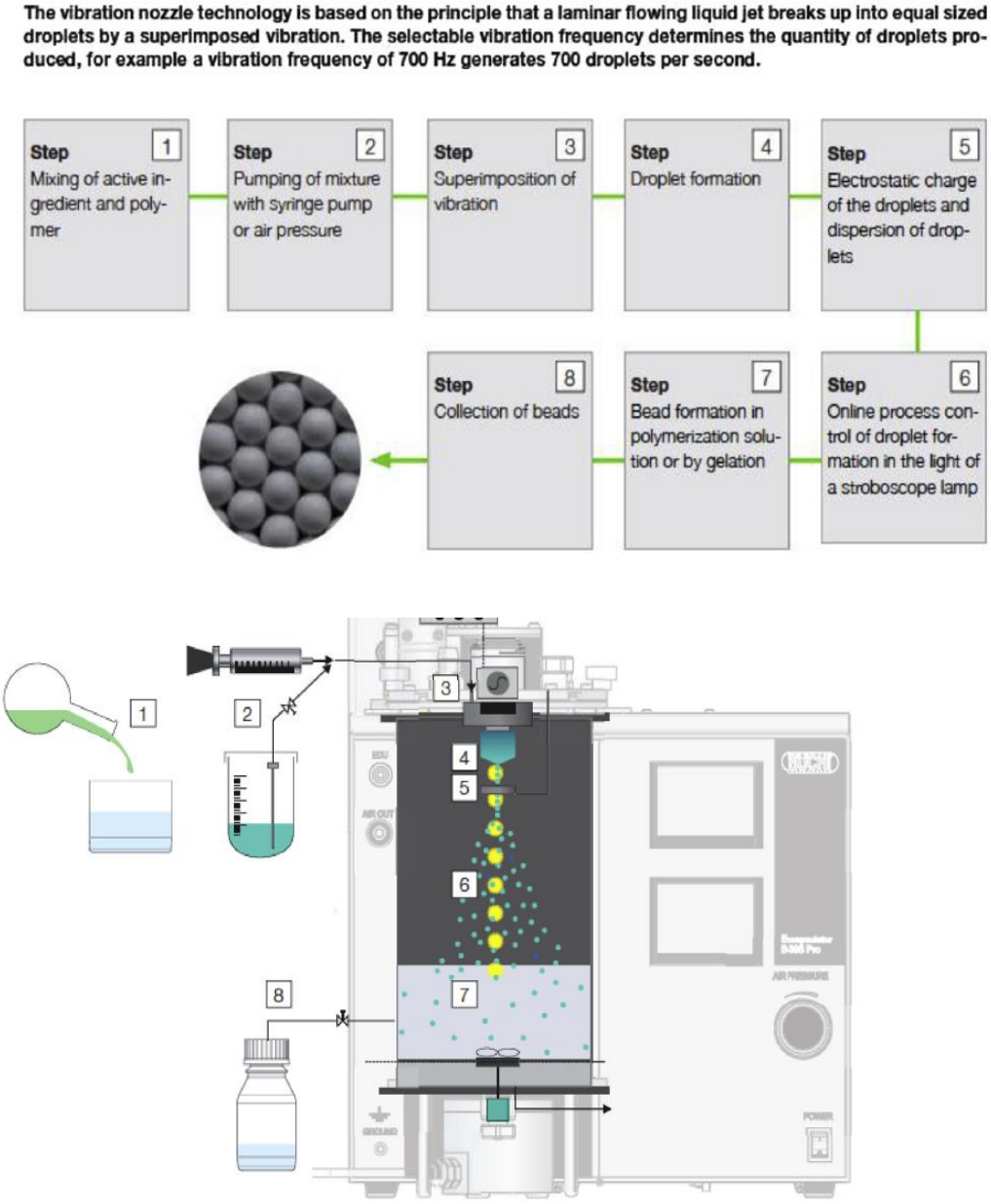

Fig 4: Steps of vibrating technology and scheme of the Encapsulator B-395 Pro (BUCHI, Switzerland).

2.5 PCR amplification of specific strain surface protein of DSM 17938

DNA, which was extracted from rat's feces and colon content by using DNeasy Tisue Kit used in identification *L. reuteri* DSM 17938 specific PCR, was identified using PuReTaq Ready-To-Go PCR Beads (GE Healthcare 27-9559-01), TBE(Tris/Borate/EDTA) buffer, standard agarose,

magnesium, ethidium bromide, loading dye, base-pair ladder (100 bp intervals) and strain-specific primers LR1/1694f: (5'-TTAAGGATGCAAACCCGAAC-3') and LR1/1694r: (5'-CCTTGTCACCTGGAACCACT-3') (Personal communication from Stephan Ross of Bio Gaia). The primer pair (both forward and the reverse primer) were mixed to the concentrations 10 pmol/25 µl (of each primer) in water. Briefly, total reaction volume of 50 µl (containing buffer 5 µl, magnesium 2.5 µl, LR1f (10 µM) 1 µl, LR1r (10 µM) 1 µl, d-NTPs (25mM) 0.5 µl, Taq 0.5 µl and sterile water) was added 4,5,10 µl of the DNA extracted. PCR condition was 30x (95°C for 30 s; 54°C for 30 s; 72°C for 30 s); 72°C for 10 min; 16°C until stopping the program. PCR products mixed with loading dye were then verified by electrophoresis on agarose gel (1% agarose, 0.5 µg/ml ethidium bromide, 0.5x TBE buffer) allowed to run for 40 min at 100 V Mix. The presence of an amplicon at about 177 bp was verified by UV illumination and the results documented.

2.6 DNA extraction and 16S rRNA gene sequencing

Fresh feces of 48 rats (8 replicates for each of six different groups) were collected at three times during the experiments (time 1 before start treatment, time 2 after 4 weeks of treatments and time 3 after 8 weeks of treatment). The colon content was squeezed out and collected separately. Both were immediately placed onto dry ice. The colon content centrifuged at (1,000 rpm X 1 min) in order to pellet debris. The supernatant was centrifuged again (12,000 X g, 2 min), and the pellet was used for DNA

extraction. The DNA was extracted by using DNeasy PowerSoil Kit (QIAGEN) (figure 5) following the manufacturer's instructions. The V3-V4 region (figure 6) of the 16S rRNA gene was amplified by using the primers S-D-Bact-0341F5'-CCTACGGGNGGCWGCAG and S-D-Bact-0785R5'-GACTACHVGGGTATCTAATCC (Klindworth et al., 2013). Each 50 µl PCR mix contained (3 µl or 4 µl or 5 µl of DNA, buffer 5 µl, enhancer 10 µl,V3(10 µM) 1 µl,V4 (10 µM) 1 µl, base-pair ladder (d-NTPs) 1 µl ,Taq 0.5 ,and sterile water). The PCR conditions used 95°C for 3 minutes, then 25 cycles of (95°C for 30 seconds,55°C for 30 seconds,72°C for 30 seconds), after that 72°C for 5 minutes, hold at 4°C.

Figure 5: DNeasy Power Soil kit for DNA extraction

The PCR products mixed with loading dye were then verified by electrophoresis on agarose gel (1.5% agarose, 0.5 µg/ml ethidium bromide, 0.5x TBE buffer) allowed to run for 40 min at 100 V Mix. the DNA fragments visualize by UV illumination and the results documented (figure 6).

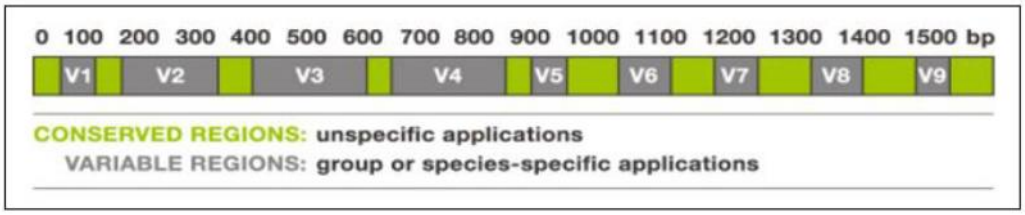

Figure 6: Scheme of the subdivision of hypervariable regions within the 16S rRNA gene sequence.

2.7 Illumina library preparation

From the total DNA, a portion of the gene coding for 16S ribosomal RNA (regions V3-V4) was amplified, resulting in an amplicon of approximately ~ 460 bp, and the space region ITS1-2, of variable size between species (200-450 bp). The primers used include, in addition to the specific sequence for the gene of interest, also a sequence that will allow the amplicon to bind to specific adapters for sequencing on the Illumina platform (Figure 7).

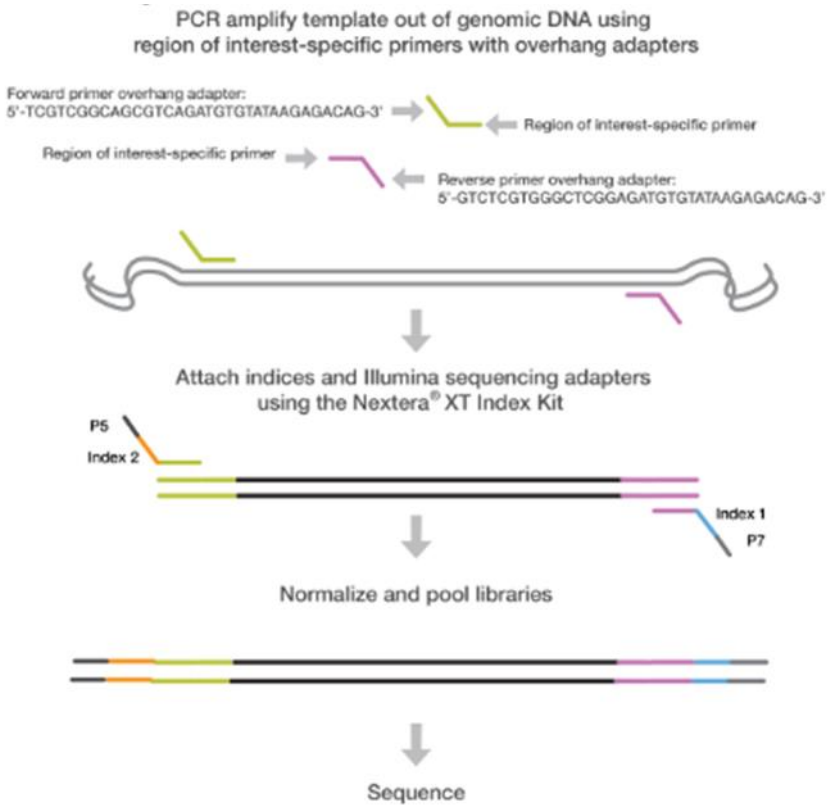

Figure 7: A pair of forward and reverse primers specific for the region of interest is designed together with an adaptive sequence: the construct is used to amplify the extracted genomic DNA as a template. A second amplification cycle is used to add the index (specific for each sample) and an adapter sequence, which allows binding to the Illumina sequencing plate. The amplicons are then normalized and joined, and subsequently sequenced with the Illumina MiSeq system.

The libraries were prepared using the Microlab STARlet workstation (Hamilton Company). The instrument is able to carry out the various purification steps with magnetic beads, quantification and normalization required for the preparation of metagenomic libraries.

2.8 Bioinformatic and statistical analysis

Demultiplexed, forward, and reverse reads were joined by using FLASH (Magoč and Salzberg, 2011). Joined reads were quality trimmed (Phred score < 20) and short reads (<250 bp) were discarded by using Prinseq (Schmieder and Edwards, 2011). High-quality reads were then imported

into QIIME (Caporaso *et al*., 2010). Operational taxonomic units (OTU) were picked using a de novo approach and the uclust method, and taxonomic assignments were obtained by using the RDP classifier and the Greengenes (McDonald *et al*., 2012) database, following a pipeline previously reported (De Filippis *et al*., 2014). In order to avoid biases due to the different sequencing depth, OTU tables were rarefied to the lowest number Data were expressed as mean values ± SEM. The program GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used to verify that raw data have normal distribution and to perform one-way ANOVA followed by Tukey post-test. A probability < 5% ($P < 0.05$) was considered statistically significant in all analyses. of sequences per sample. Bray-Curtis distance matrix and alpha diversity indices were computed by QIIME on rarefied OTU tables. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (http://picrust.github.io/picrust) (Langille *et al*., 2013) was used to predict the functional profiles of the samples, as recently reported (De Filippis *et al*., 2016). Statistical analyses and plotting were carried out in an R environment (https://www.r-project.org). Permutational multivariate analysis of variance (nonparametric MANOVA) based on Jaccard and Bray-Curtis distance matrices was carried out using 999 permutations to detect significant differences in the overall microbial community or oligotype patterns, by using the adonis function in the vegan package. The Bioconductor statistical package DeSeq2 (Love *et al*., 2014) was used to find taxa differentially abundant between the groups. Spearman’s pairwise

correlations were computed between OTU and other quantitative variables (the corr.test function in the psych package) and plotted by using the heatplot function in the made4 package. P values were corrected for multiple testing using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

Data for body composition, energy balance and glucose homeostasis were expressed as mean values ± SEM. The program GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used to verify that raw data have normal distribution and to perform one-way ANOVA followed by Tukey post-test. A probability < 5% ($P < 0.05$) was considered statistically significant in all analyses.

Chapter Three

Results

3.1 Microencapsulated L. reuteri improved glucose homeostasis in the HFF diet -induced rats

In order to have general picture of glucose balance homeostasis, plasma glucose levels during GTT analysis were evaluated. After 8 weeks of treatment, HFF fed rats exhibited significantly higher values of plasma glucose compared to the control rats, while the TR and TRM groups showed values comparable to the control. (Figure 8).

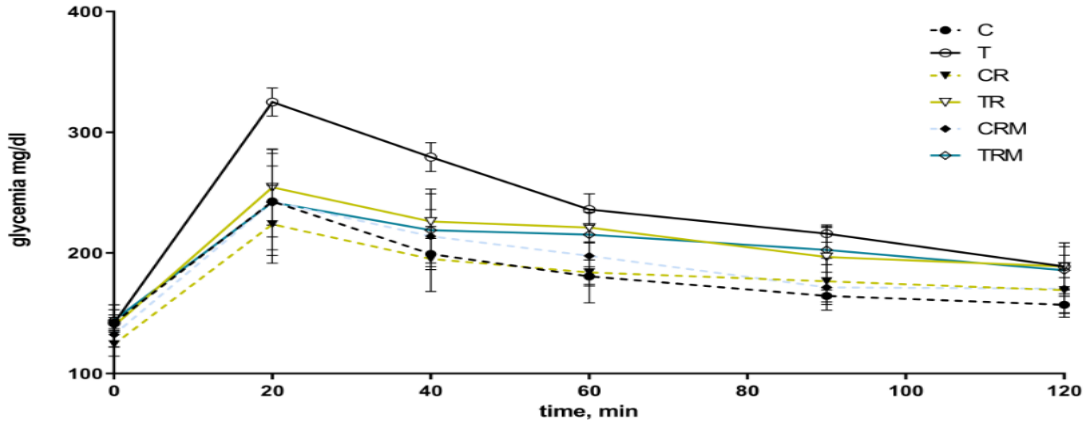

Fig 8: L.reuteri (free and microcapsulated) improved glucose homeostasis in HFF diet-induced obese rats. Serum glucose levels at different time-points in oral glucose tolerance tests (GTT). Values are the means ± SEM of eight different rats. * p < 0.05 compared to respective control (one-way ANOVA followed by Tukey post-test).

The area under the curve (AUC) was significantly higher in the HFF group (T) than in the normal group (C) . Nevertheless, treatment of the HFD group with L.reuteri (free and microcapsules) reduced the levels of this parameter comparing to HFF group (T) (figure 9).

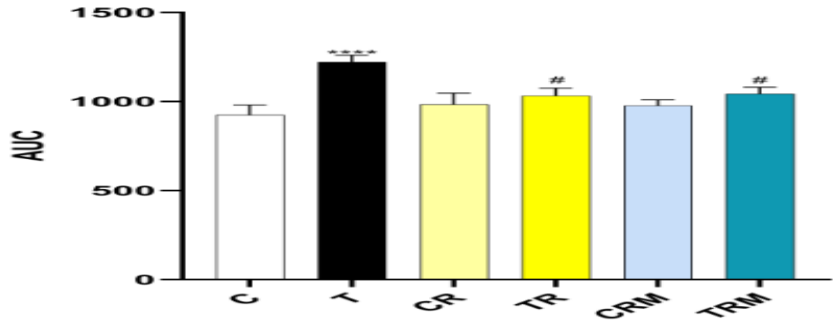

Fig 9: L.reuteri improved glucose homeostasis in HFF diet induced obese rats. Areas under the curve of GTT measured between 0 and 120 min after glucose administration. Values are the means ± SEM of eight different rats. **** p < 0.0001 compared to respective control (one-way ANOVA followed by Tukey post-test).(#:TR,TRM vs T)

3.2 Body composition and energy balance

High fat fructose diet-induced obese rats were used in this experiment to evaluate the role of free and microencapsulated L. reuteri DSM 17938 on body composition and energy balance. The initial body weight for all rats were almost the same (figure 10 A). At the end of the experiment the final body weight for HFF rats groups treated with L. reuteri free and microencapsulated (T, TR, TRM) were almost the same, but significantly higher comparing to their control groups (C, CR, CRM) (figure 10 B).

It was observed that rats treated with high fat fructose diet supplemented with free and microencapsulated L. reuteri significantly exhibited higher body weight gain after 8 weeks (figure 10 C) comparing to controls. The HFF diet group (T) showed a significant increase in body energy (figure 10 G), and body lipid (figure 10 D), without any significant change in body water (figure 10 F) and body protein (figure 10 E) (g/100g) between all groups.

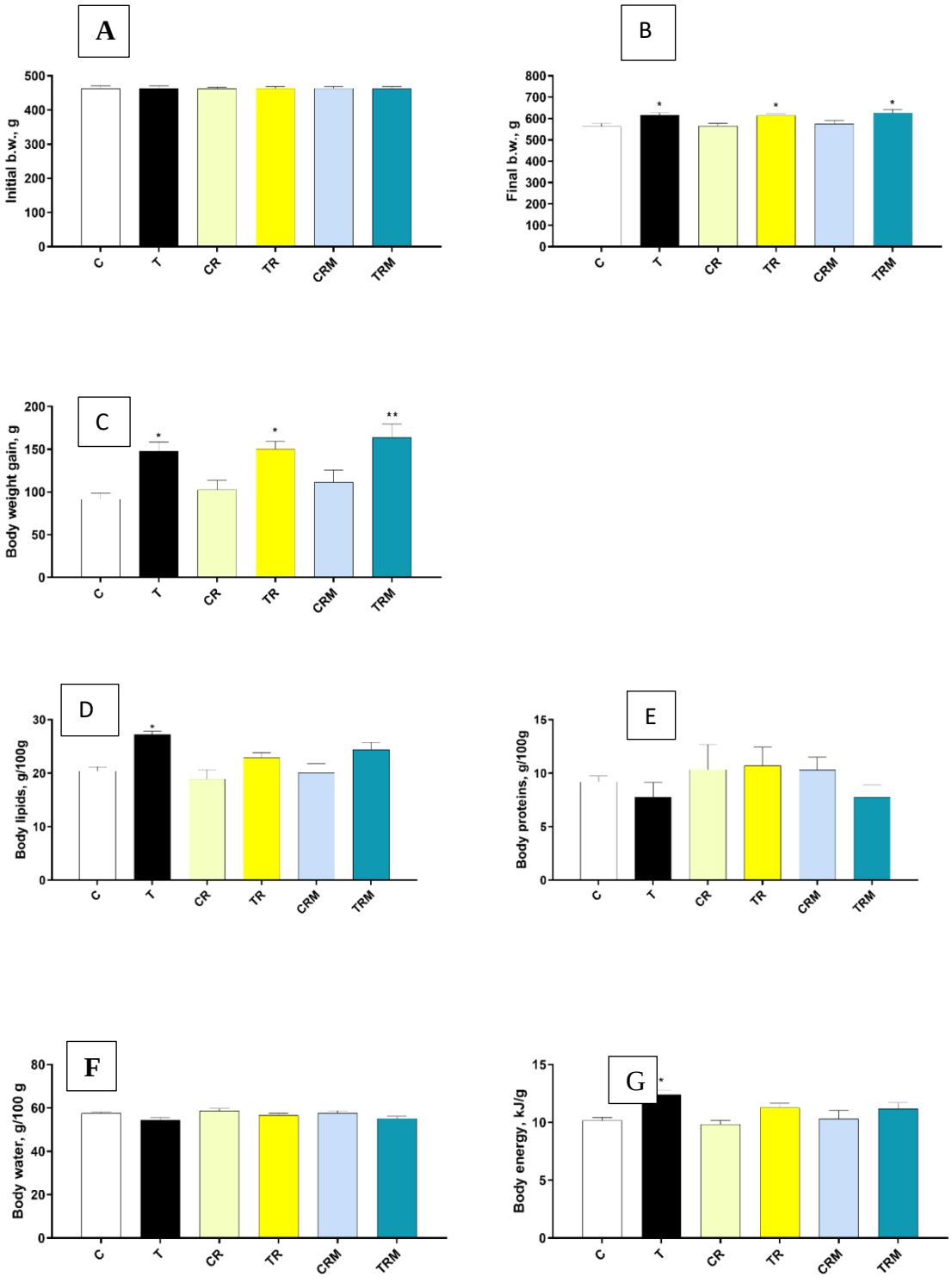

Figure 10 : Body compostion .Initial body weight (A), final body weight (B) ,body weight gain (C),body lipids (D),body protiens (E),body water (F) and body energy (G) of high fat fructose diet fed group, high fat fructose diet with microencapsulated and free L.reuteri - supplemented rats groups and their counterparts after eight-week treatment (n = 8 for each group). Values are the means ± SEM of eight different rats.* $p < 0.05$, $p < 0.01$ compared to respective control (one-way ANOVA followed by Tukey post-test).**

Body energy gain results showed a significant increase in HFF fed rats while no significant changes in HFF with microencapsulated L.reuteri rats group comparing to controls (figures 11 H).Also body lipid gain was significantly higher in group treated with HFF diet comparing to their control(figure 11 I)

Body protiens gain results (calculated as the difference between body energy gain and lipid energy gain and expressed in KJ) indicated a no significant diffrences between the groups treated HFF diet (T ,TR ,TRM) and their control groups (C , CR , CRM) (figure 11 J).

Gross energy intake KJ was significantly higher in HFF diet fed groups (T, TR, TRM) compare to their controls (C, CR, CRM) (figure 11 K), whereas food intake did not change (figure 11 L).

Since changes in energy intake are the primary drive of obesity development, metabolisable energy (ME) intake was monitored throughout the experimental period to verify whether the fat fructose-induced affect body energy and lipid content due to an change of ME. To this aim, food intake and energy loss through faeces were analyzed and indicated that ME intake was similar in all high fat fructose groups (T ,TR ,TRM), but significantly different from control groups (C, CR, CRM) (figure 11 M). Energy expenditure was significantly increased ($p < 0.05$) after supplemntation with microencapsulated and free L.reuteri in HFF diet group comparing to their control (figure 11 N).

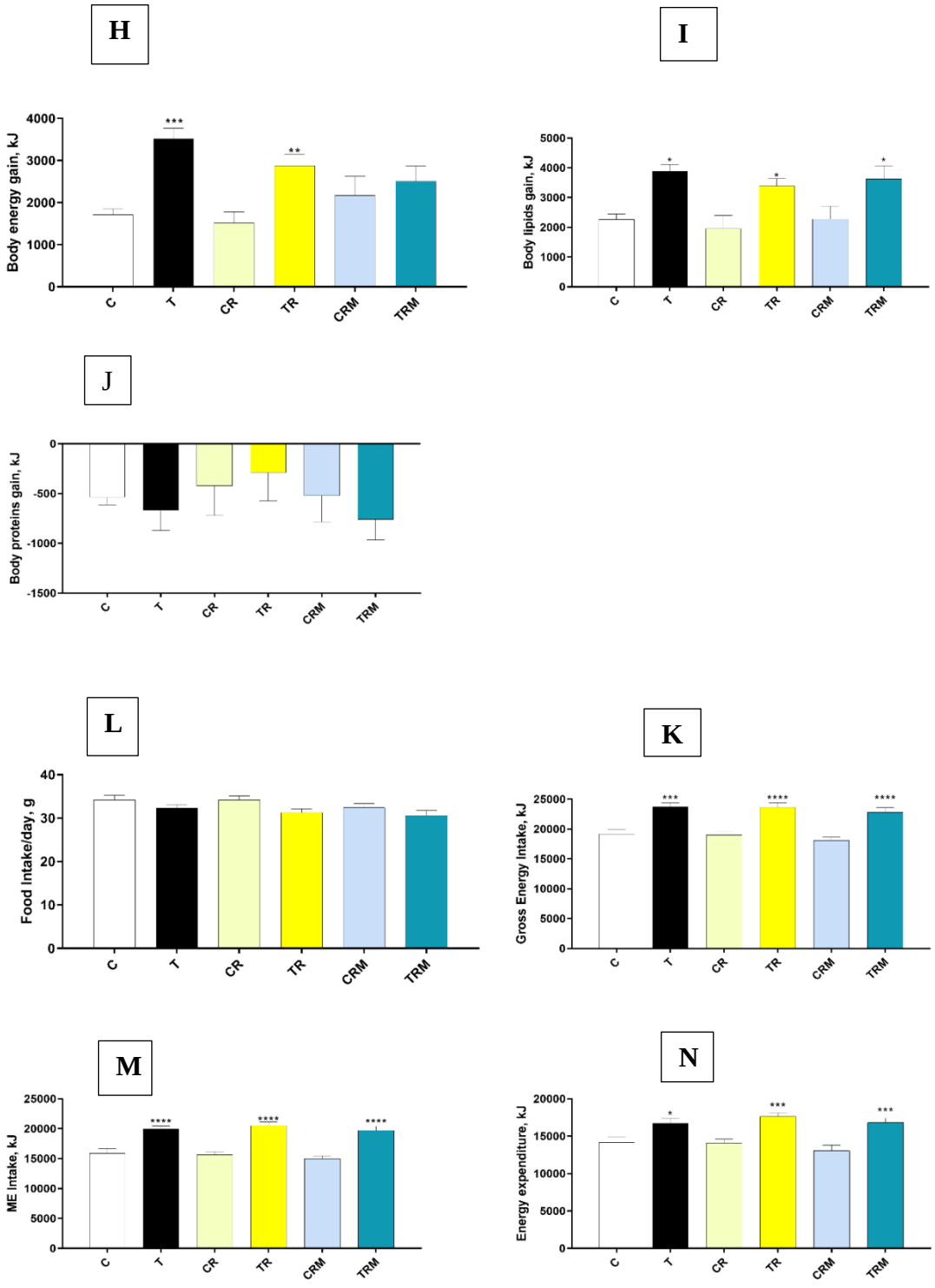

Figure 11: Energy balance. Body energy gain (H), body lipids gain (I), body proteins gain (J), food intake (L), gross energy intake (K), metabolizable energy intake (M) and energy expenditure (N) of high fat fructose diet fed group, high fat fructose diet with microencapsulated and free L. reuteri -supplemented rat's groups and their counterparts after eight-week treatment (n = 8 for each group). Values are the means ± SEM of eight different rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to respective control (one-way ANOVA followed by Tukey post-test).

3.3 Microencapsulated L.reuteri attenuated high fat-fructose diet-induced hepatic steatosis

High fat diet-fructose fed rats showed significantly higher levels of serum ALT and AST than those of control diet-fed rats (*** $p < 0.001$ for AST ,and **** $p < 0.0001$ for ALT). To determine whether microencapsulated L.reuteri treatment could mitigate the HFD-induced liver injury, the concentrations of these enzymes were examined. The HFF diet-induced elevation in serum AST and ALT were significantly prevented by microencapsulated L.reuteri administration in HFF-fed rats (figure 13). Assessment of hepatic steatosis by quantififcation revealed that after 8 weeks of fat fructose-rich diet the steatosis (Figure 13 C), significantly increased in HFF rats, while all these modifications were completely reversed after treatment with microencapsulated L.reuteri.

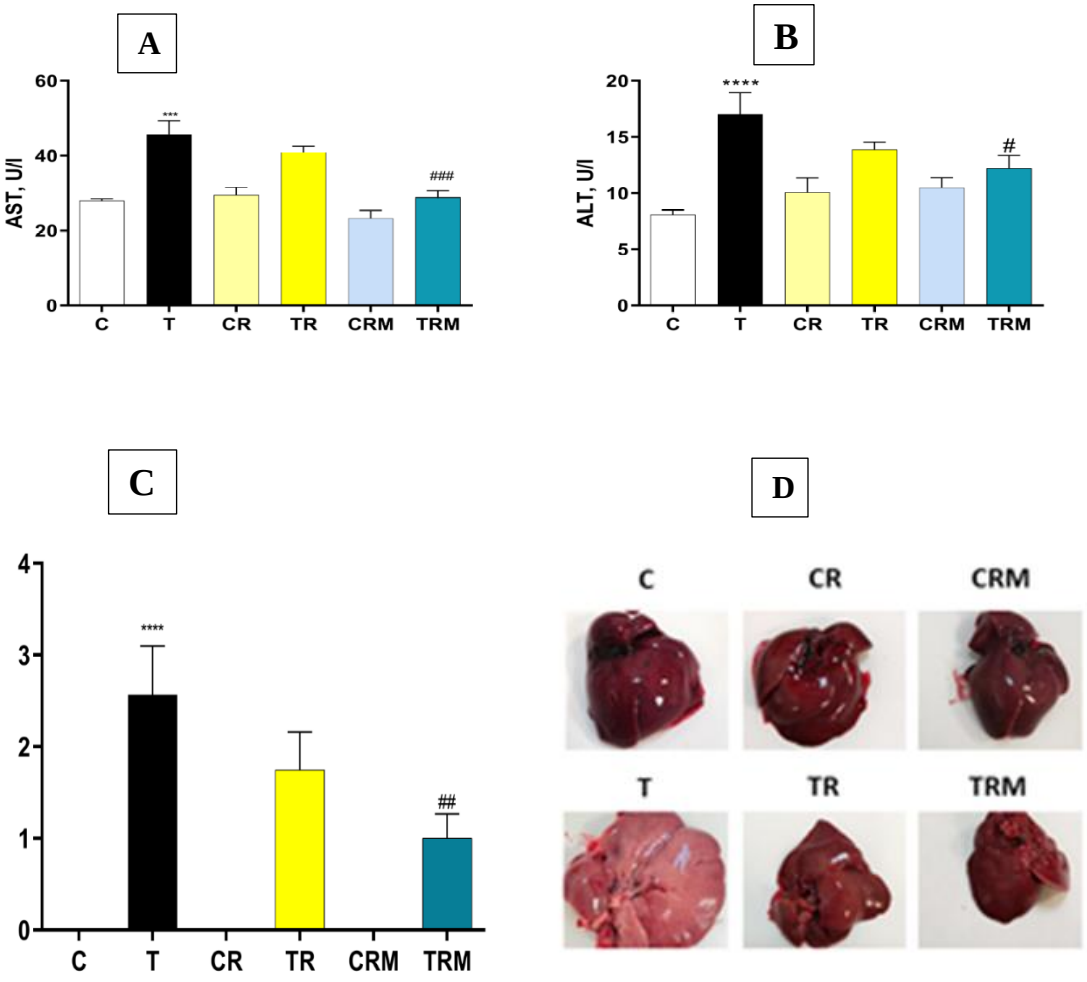

Figure 12 :Effect of microencapsulated L.reuteri on liver enzymes, (A) AST serum level, (B) ALT serume level ,(C) liver quantification (D) liver images .Values are the means ± SEM of eight different rats. *** p < 0.001 , **** p < 0.0001 compared to respective control (one-way ANOVA followed by Tukey post-test). (#:TR,TRM vs T)

3.4 The effect of L.reuteri adminstration on the intestinal microbiota composition in the HFF-Induced Rats

To profile the effects of diet and L. reuteri on microbiota composition, we performed sequencing of bacterial 16S rRNA gene V3 and V4 regions for 144 fecal samples collected from 48 rats at baseline, after 4 weeks, and after 8 weeks. The overall microbial composition of the gut of rats of groups C, CR and CRM at the genus level were altered by the different

treatments (free L. reuteri and ME L. reuteri) ($p < 0.05$) in the three different times.The most abundant genera were colostridia which was the same in all groups and different times, Verrucomicrobia- akkermansia muciniphila and prevotellaceae were more abundant at baseline in all groups implemented control diet and treated with free or microencapsulated L.reuteri and these two genera have been shown to decrease with the time (figure 13). In addition, the microbial composition of the gut of the rats of groups T,TR,TRM at the genus level were altered by the different treatments (free L. reuteri and ME L. reuteri) ($p < 0.05$) in the three different times. Prevotellaceae showed decrease with the time, colostridia did not change with time in different groups but Verrucomicrobia-akkermansia muciniphila significantly increase in groups during the time (Figure 14).

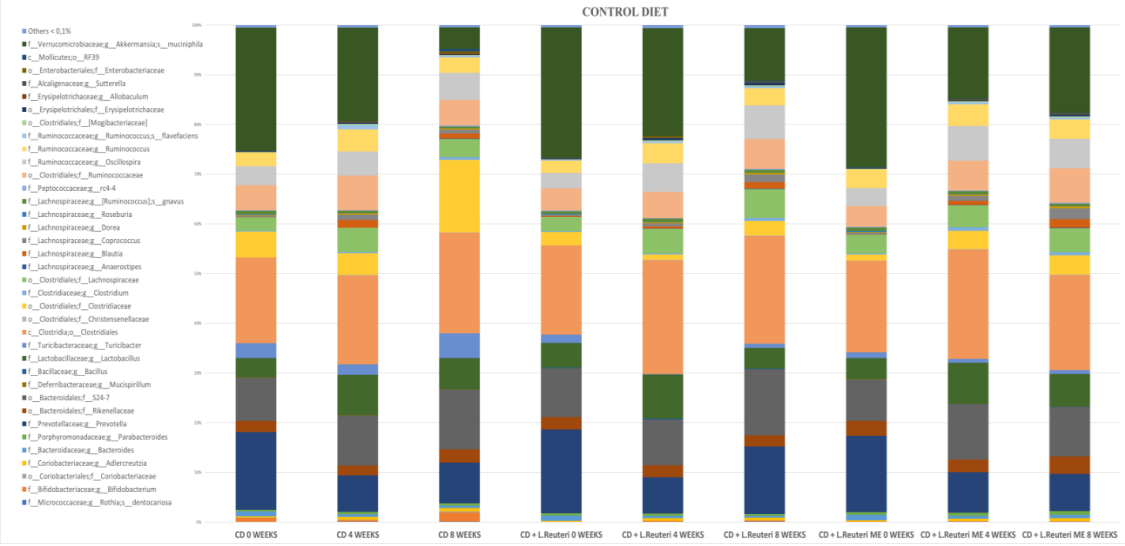

Figure 13: Relative Operational Taxonomic Units (OTUs) abundance at the genus level in control diet (C), control diet with *L. reuteri* (CR), and control diet with microencapsulated *L. reuteri* (CRM) rats in three different time. Composition of fecal microbiota of rats from different groups as revealed by Illumina sequencing of V3-V4 hypervariable region of 16S rRNA gene. Population analyses for each group show phylotypes at genus level and are reported as means of eight rats for each group ($p < 0.05$).

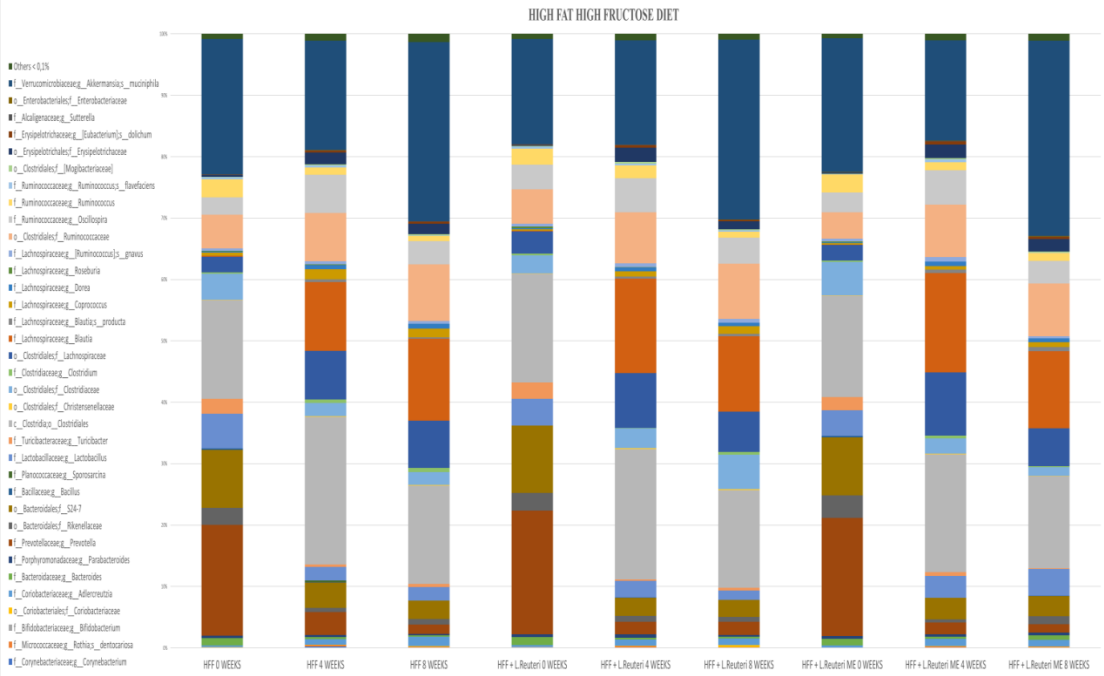

Figure 14: Relative Operational Taxonomic Units (OTUs) abundance at the genus level in high fat fructose diet (T), high fat fructose diet with L. reuteri (TR), and high fat fructose diet with microencapsulated L. reuteri (TRM) rats in three different time. Composition of fecal microbiota of rats from different groups as revealed by Illumina sequencing of V3-V4 hypervariable region of 16S rRNA gene. Population analyses for each group show phylotypes at genus level and are reported as means of eight rats for each group ($p < 0.05$).

Chapter Four
Discussion and Coclusion

4.1 Discussion

We observed a statistically significant increase in body weight in HFF diet fed groups supplemented with microencapsulated or free L. reuteri DSM 17938. In fact, this increase was reflected the increase in energy intake due to HFF diet consumption. The analysis of the administration of microencapsulated L.reuteri on HFF diet rats groups on energy balance and other body composition showed no significant changes, as a matter of fact, we don’t suspect that probiotic bacteria decrease body weight ,fat gain or energy gain specially in presence of high fat fructose diet which is needed to show how the composition of this diet affect on these parameters. One another hand, one systematic review, found that probiotic effect in body weight is specie and strain specific.For instance L. gasseri BNR17, reduced the weight gain compared to controls ,L. gasseri L66-5 promoted weight gain,while L. rhamnosus GGMCC is the only one that had a positive effect in weight loss in humans (Rouxinol-Dias *et al*.,2015). This discrepancy might be due to the difference in strain, length of the treatment ,diet content , or the target population.

Recently, it was found that, in adult rats, long-term feeding a high-fructose and high-fat diet elicits the development of obesity and insulin resistance (Crescenzo *et al*.,2013; Lionetti *et al*.,2007) .Also growing evidence supports the crosstalk between gut microbiota and host health, and

alterations in the composition of gut microbiota are involved in the development of metabolic disorders such as obesity and T2DM (Hartstra *et al*. 2015). Thus, elucidating the effects of candidate bacterial species on host metabolism will provide new methods for preventing and treating metabolic diseases. In this study, we examined the metabolic benefits of L.reuteri in rats fed HFF diet. Interestingly, we found that L.reuteri daily gavage for eight weeks significantly improved plasma glucose. In fact, Gut microbiota ultimately contribute to the regulation of incretin hormone secretion through the interaction between the aforementioned metabolites (SCFAs, bile acids) and their receptors (GPR1 and GPR43, TGR5), which are expressed on enteroendocrine L cells (Osborn & Olefsky,2012; Caesar et al.,2015; Feuerer et al.,2009). The stimulated enteroendocrine L cells secrete incretin hormone peptides, such as glucagonlike peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), and peptide YY (PYY), which in turn stimulate insulin release and decrease blood glucose levels (Drucker & Nauck, 2006). These secreted peptides affect a wide range of organs and tissues to improve insulin sensitivity, glucose tolerance, and energy homeostasis, thereby contributing to protection in metabolic disorders such as obesity and type 2 diabetes (Yang& Kweon, 2016). Also we can say, despite the HFF rats groups increase their body weight, they still have healthy metabolic parameters (plasma glucose and insuline resistance) .Finally these result were inconsistancy with Mobini et al. in 2017 revelaed that oral supplementation with Lactobacillus reuteri DSM 17938 in patients

with type 2 diabetes for 12 weeks improved insulin sensitivity and glucose homeostasis (Mobini *et al*.,2017).

In the past decade, research on probiotics has attracted much attention due to their protective role in NAFLD(Azarang *et al*.,2020). Plasma levels of ALT and AST were assessed as marker of liver damage(Yin et al.,2012). NKT cells regulation of inflammatory processes and chronic inflammation is known to be associated with insulin resistance and fatty liver disease(Yuan et al.,2001). In the present study, we demonstrated that microencapsulated L.reuteri effectively prevents HFF diet-induced hepatic steatosis in rats.One study reported that a high fat diet that induces obesity, insulin resistance and hepatic steatosis also leads to hepatic NKT cell depletion. However, it was not clear whether the depletion of hepatic NKT cells was the cause or the consequence of overall metabolic dysfunction(Ma *et al*.,2008). Cani and colleagues reported that a high fat diet increases plasma lipopolysaccharide (LPS) level, which also contributes to the pathogenesis of insulin resistance and increased liver triglyceride content (Cani *et al*.,2007) .It is possible that the endotoxinemia caused by high fat diet reduces intrahepatic NKT cells(Ma *et al*.,2008).

The ability of probiotics to restore hepatic NKT cells and improve HF diet-induced insulin resistance and fatty liver are novel findings and intriguing (Ma et al.,2008).And that findings were inconsistancy with our hypothesis that microencapsulated L.reuteri modulation of hepatic NKT cells, reduce inflammatory signaling and ultimately lead to improved insulin resistance

and to improved fatty liver disease.NKT cells regulation of inflammatory processes and chronic inflammation is known to be associated with insulin resistance and fatty liver disease.

Metabolic disorders were characterized by intestinal inflammation and mucosal-barrier dysfunction, which facilitate the translocation of luminal toxicity into the host (Winer *et al*.,2016). An HFD clearly damages intestinal barrier function and downregulates the major goblet cell mucin producing gene Muc2, antimicrobial gene Reg3g (Chang *et al*.,1996) ,and tight junction proteins ZO-1 and occludin in mice (Rahman *et al*.,2016). A high fat-fructose diet on male Wistar rats for 4 weeks decreased the abundance of *A. muciniphila*, but it increased again after 8 weeks. While, supplementation with microencapsulated *L. reuteri* on the high fat-fructose diet was able to increase the abundance of *A. muciniphila* more than the high fat fructose diet alone. The exact mechanisms by which A. muciniphila exerts the beneficial impact on health have not been fully elucidated. The positive modulation of mucus thickness and gut barrier integrity by *A. muciniphila* could be the key (Zhou,2016). *A. muciniphila* supplementation was able to restore mucus thickness in obese and type 2 diabetic mice where gut mucus was disrupted by high fat diet treatment (Everard et al., 2013).Although how *A. muciniphila* could promote mucus thickness is not known. One of the reasons could be A. muciniphila stimulates mucus turnover rate by making short-chain fatty acids from the degraded mucin, the preferable energy sources for the host epithelium which synthesize and secret mucin (Zhou,2016).

4.2 Coclusion

This study strongly suggests that supplementation with microencapsulated L.reuteri DSM 17938 is an effective probiotic bacterium in decreasing plasma glucose, improving insulin sensitivity and glucose homeostasis,hepatic homeostasis and improving gut microbiota specially in high fat fructose diet without changes on body compostion and energy balance. However, there is a need for further investigation on this L.reuteri strain, especially on humans.

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تأثير التزويد اليومي لليموسيلاكتوباسلس ريوتيري DSM 17938 المتحوصلة على ميكروبيوتا الامعاء والتغيرات الأيضية محفز بنظام غذائي عالي بالدهون والفركتوز في الجرذان البالغة

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الملخص

في الآونة الحديثة تغيرت البيئة والسلوكيات ونمط الحياه البشرية بشكل كبير وقد ارتفعت معدلات السمنة ومتلازمة التمثيل الغذائي نتيجة لهذه التغيرات. بالإضافة إلى ذلك، فهي تمثل مخاوف صحية عامة كبيرة بسبب انتشارها العالمي وارتباطها بارتفاع مخاطر الإصابة بالأمراض المزمنة.

نظرًا لأن السمنة هي السالف الرئيسي لمتلازمة التمثيل الغذائي التي يمكن استهدافها في تطوير علاجات مختلفة، فقد تم اقتراح العديد من العلاجات الجسدية والنفسية والصيدلانية والغذائية لإدارة هذه المشكلة. ومع ذلك، فقد ثبت أن الممارسات الغذائية أكثر فعالية دون التسبب في أي آثار صحية سلبية. تعتبر إدارة البروبيوتيك كعلاجات حيوية مجالًا جديدًا نسبيًا في تطوير الأساليب الغذائية، وكثير من الناس لديهم فضول حول العلم الكامن وراء هذه الممارسات الصحية.

تهدف هذه الرسالة الى التحقيق في تأثير التزويد اليومي لليموسيلاكتوبسلس ريوتيري 17938 DSM المتحوصلة على ميكروبيوتا الامعاء والتغيرات الايضيه محفز بنظام غذائي عالي بالدهون والفركتوز في الجرذان البالغة.

وللحصول على نتائج هذه الدراسة في تجربة على الحيوانات، تم تغذية ذكور فئران ويستار لمدة 90 يومًا بنظام غذائي تحكم أو نظام غذائي غني بالدهون مع نسبة عالية من الفركتوز HFF لمدة 8 أسابيع، مما يعزز علامات السمنة ومتلازمة التمثيل الغذائي. تم تقسيم الفئران التي تتغذى على نظام غذائي تحكم إلى ثلاث مجموعات. المجموعة الأولى، المجموعة الضابطة (C) وهو نظام

غذائي قليل الدسم، والثانية، نظام غذائي قليل الدسم مع اعطاء الليموسيلاكتوبسلس ريوتيري داخل الكبسولة الدقيقة (CRM)، في حين تم تلقي المجموعة الأخيرة نظام غذائي قليل الدسم مع اعطاء الليموسيلاكتوبسلس ريوتيري (CR)، تم تقسيم الفئران التي عولجت بنظام غذائي عالي الدهون وفركتوز (HFF) إلى ثلاث مجموعات: واحدة نظام غذائي عالي الدهون وفركتوز فقط (T)، المجموعة الثانية خضعت لليموسيلاكتوبسلس ريوتيري داخل الكبسولة الدقيقة (TRM)، والمجموعة الثالثة عولجت بنظام غذائي عالي الدهون وفركتوز مع اعطاء الليموسيلاكتوبسلس ريوتيري (TR). يبحث البحث في تأثير المكملات على توازن الجلوكوز وتكوين الجسم وتوازن الطاقة والتشحم الكبدي وتكوين البكتيريا البرازية.

بناء على التحليل الاحصائي للنتائج تبين ان المكملات باستخدام الكبسولات الدقيقة الليموسيلاكتوبسلس ريوتيري DSM 17938 لمدة 8 أسابيع لم يؤثر بشكل كبير على تكوين الجسم وتوازن الطاقة ولكنها تؤثر بشكل كبير على مقاومة الجلوكوز والأنسولين في البلازما، والتنكس الدهني للكبد، وتركيب البكتيريا المعوية. أظهرت الجرذان التي تلقت الكبسولة الدقيقة انخفاضًا في جلوكوز البلازما مقارنةً بمجموعة التحكم. بالإضافة إلى ذلك، فإن مكملات الليموسيلاكتوبسلس ريوتيري المتحوصله تثبط بشكل كبير إنزيمات الكبد (AST، ALT) والتنكس الدهني الكبدي، كما تزيد من وفرة بكتيريا الأكريمانسيا موشنيفيلا مما يحسن معاملات التمثيل الغذائي للمضيف.