TATIANA FICHE SALLES TEIXEIRA

PERMEABILIDADE INTESTINAL E PARÂMETROS NUTRICIONAIS E BIOQUÍMICOS NA OBESIDADE

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Gradução em Ciência da Nutrição, para obtenção do título de *Magister Scientiae.*

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APROVADA: 17 de dezembro 2010

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RESUMO

TEIXEIRA, Tatiana Fiche Salles. M.Sc. Universidade Federal de Viçosa, Dezembro de 2010. **Permeabilidade intestinal e parâmetros nutricionais e bioquímicos na obesidade.** Orientadora: Maria do Carmo Gouveia Peluzio. Co-orientadoras: Josefina Bressan e Célia Lúcia de Luces Fortes Ferreira.

A importância da saúde intestinal para o controle de peso e prevenção da obesidade tem recebido maior atenção recentemente. O intestino abriga uma complexa estrutura celular, imunológica e neuroendócrina capaz de modular mecanismos relacionados à obesidade. O objetivo do presente estudo foi avaliar a permeabilidade intestinal de voluntárias eutróficas e obesas e discutir os possíveis mecanismos que expliquem alteração da permeabilidade intestinal na obesidade e sua ligação com comorbidades relacionadas a este estado nutricional. Vinte mulheres eutróficas (IMC 19-24.99 kg/m^2) e vinte mulheres obesas (IMC >30 kg/m^2) de idade semelhante (média de idade das mulheres magras e obesas 28.5±7.6 *vs* 30.7 ± 6.5, p=0.33) participaram do estudo. Composição corporal, análises bioquímicas, ácidos graxos de cadeia curta (AGCC) fecal e o teste de permeabilidade intestinal com o uso de lactulose e manitol foram realizados. A pressão arterial e a glicose sanguínea, embora entre os limites de normalidade, foram maiores no grupo das obesas ($p<0.05$). Baixa concentração de HDL e altos valores de insulina e índice HOMA foram observados nas obesas ($p<0.05$). A excreção de lactulose mostrou-se aumentada nas obesas ($p<0.05$), enquanto a de manitol mostrou tendência aumentada ($p=0.06$). A razão L/M pode não ser o marcador ideal para a permeabilidade intestinal alterada porque indivíduos obesos podem estar absorvendo proporcionalmente maiores quantidades de ambos os açúcares usados no teste. A concentração de insulina e o índice HOMA apresentaram correlação positiva com a excreção urinária de manitol e lactulose e com a razão L/M ($p<0.05$). As circunferências da cintura e abdominal também correlacionaram positivamente com a excreção de lactulose ($p<0.05$). A concentração de HDL foi negativamente relacionada à razão L/M ($p<0.05$). A mediana da concentração dos ácidos butírico, propiônico, acético nas fezes do grupo de obesas foi respectivamente 94.3%, 144.5% e 106.8% maior do que no grupo das eutróficas e a proporção individual dos ácidos graxos de cadeia curta alterou em favor do propionato no grupo de obesas. Foi encontrada correlação significativa ($p<0.05$) entre AGCC e fatores de risco de síndrome metabólica como baixa concentração de HDL, circunferência da cintura aumentada e índice

HOMA. Disbiose e deficiências nutricionais podem ser apontadas como possíveis mecanismos principais relacionados à alteração da permeabilidade intestinal, e mudanças na concentração e proporção individual de AGCC são relacionados com mudanças na composição da microbiota intestinal. Estudos sugerem que a endotoxemia, a partir da permeação intestinal alterada de LPS, pode explicar algumas conseqüências da obesidade como esteatose hepática de origem não alcoólica, resistência insulina, baixos níveis de HDL e hiperleptinemia. Em resumo os nossos resultados estão de acordo com relatos anteriores que sugerem que produção aumentada de AGCC e permeabilidade intestinal alterada podem desempenhar um papel importante na obesidade. A proteção da barreira intestinal como abordagem preventiva ou terapêutica é uma área de pesquisa clínica e experimental raramente explorada, mas que guarda um campo promissor para o futuro desenvolvimento de intervenções inovadoras na obesidade.

ABSTRACT

TEIXEIRA, Tatiana Fiche Salles. M.Sc. Universidade Federal de Viçosa, December, 2010. **Intestinal permeability and nutritional and biochemical parameters in obesity.** Advisor: Maria do Carmo Gouveia Peluzio. Co-advisors: Josefina Bressan and Célia Lúcia de Luces Fortes Ferreira.

The importance of gut health for weight control and prevention of obesity has recently gained more attention. The gut harbors a complex cellular, immunological and neuroendocrine structure that can modulate mechanisms related to obesity. The aim of this study was to assess intestinal permeability in lean and obese female volunteers and to discuss possible mechanisms underlying altered intestinal permeability in obesity and its link with obesity-related comorbidities. Twenty lean (BMI 19-24,99 kg/m^2) and twenty obese females (BMI > 30 kg/m^2) of matched age (mean age of lean and obese group 28.5±7.6 *vs* 30.7 ± 6.5, p=0.33) participated in this study. Body composition, biochemical analyses, fecal short-chain fatty acids and intestinal permeability test using lactulose and mannitol were measured. Blood pressure and blood glucose, although within the normal limits, were higher in the obese group ($p<0.05$). Low-HDL concentration and high insulin and HOMA index values were observed in the obese group ($p<0.05$).Lactulose excretion was shown to be increased in obese individuals ($p<0.05$), while mannitol tended to be also increased ($p=0.06$). The L/M ratio might not be the best marker of altered intestinal permeability because obese individuals might be absorbing proportionally higher quantities of both sugar probes. Insulin concentration and HOMA index showed positive correlation with mannitol and lactulose excretion and L/M ratio ($p<0.05$). Waist and abdominal circumference have shown positive correlation with lactulose excretion ($p<0.05$). HDL concentration was negatively related to L/M ratio ($p<0.05$). The median values of butyric, propionic and acetic acid in the obese group was respectively 94.3%, 144.5% and 106.8% higher in comparison with the lean group and the proportion of individual SCFA changed in favor of propionate in obese subjects. It was found significant correlation between SCFA and metabolic syndrome risk factors such as low HDL, increased waist circumference and HOMA index ($p<0.05$). Dysbiosis and nutritional deficiencies can be pointed out as the possible main mechanisms underlying the causes of altered intestinal permeability, and changes in concentration and proportion of individual SCFA are concurrent with changes in

intestinal microbiota composition. Studies suggest that endotoxemia, through altered intestinal permeation of LPS, might explain some consequences of obesity such as non-alcoholic fatty liver, insulin resistance, low HDL levels and hyperleptinemia. In summary our results are in line with previous reports suggesting that increased SCFA production and altered intestinal permeability might play a considerable role in obesity. Protection of intestinal barrier as a preventive or therapeutic approach is an area of clinical and experimental research barely explored, but that harbors a promising field for future development of innovative interventions in obesity.

ARTIGO I:

Altered intestinal permeability in obesity: what evidences and possible mechanisms support this hypothesis?

Abstract

The importance of gut health for weight control and prevention of obesity has recently gained more attention. The gut harbors a complex cellular, immunological and neuroendocrine structure that can modulate mechanisms related to obesity. The aim of this review was to discuss mechanisms underlying possible altered intestinal permeability in obesity and its link with obesity-related comorbidities. The L/M ratio might not be the best marker of altered intestinal permeability because obese individuals might be absorbing proportionally higher quantities of both sugar probes, and this need to be explored. Dysbiosis and nutritional deficiencies can be pointed out as the main mechanisms underlying the causes of altered intestinal permeability. Studies evidences suggest that endotoxemia, through altered intestinal permeation of LPS, might explain some features of obesity such as non-alcoholic fatty liver, insulin resistance, low HDL levels and hyperleptinemia. Protection of intestinal barrier as a preventive or therapeutic approach is an area of clinical and experimental research barely explored, but that harbors a promising field for future development of innovative interventions.

Key words: intestinal permeability, obesity, dysbiosis, endotoxemia

1. Introduction

Many countries around the world have been experiencing increases in the prevalence of obesity since 1980s (James et al, 2001; Finkelstein et al, 2005; Ogden et al, 2007; Ford & Mokdad, 2008). Obesity can be defined as a disease in which excess body fat has accumulated such that health may be adversely affected, explaining why it is a medical and public health concern (Kopelman, 2000). It is considered a major risk factor for cardiovascular disease, once it is also related to hypertension, dyslipidemia and insulin resistance, and it is associated with increased risk of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (Ogden et al, 2007).

The combination of genetic susceptibility (Cummings & Schwartz, 2003), decreased physical activity, increased consumption and availability of high-energy foods in modern society are blamed as the main causes of this global epidemic of obesity (Kopelman, 2000; Finkelstein et al, 2005).

Although obesity is considered a multifactorial condition, it is often viewed unidimensionally, described and studied as a simple issue of body weight (Ogden et al, 2007). But recently, the importance of gut health for weight control and prevention of obesity has gained more attention. The autointoxication theory guided the medical practice until the early twentieth century. This theory stated that toxins produced in the intestine by bacterial processing could be absorbed to the circulation and promote many symptoms and diseases (Whorton, 2000; Müller-Lissner et al, 2005). Thus, accumulating evidences point out to the need of, at least partially, reconsidering this old concept.

The gut harbors a complex cellular, immunological and neuroendocrine structure that can modulate mechanisms related to obesity. For example, the gut-brain axis plays a role in the control of food intake (Konturek et al, 2004; Murphy et al, 2006) and exciting evidences suggests that gut microbiota might be part of the picture of obesity, although it is not yet defined whether as a cause or consequence (Bäckhed, 2009). Since there are clearly documented associations of obesity (especially abdominal obesity) with a number of gastrointestinal disease risk factors and outcomes such as colon cancer, non-alcoholic fatty liver, acute pancreatitis and gall bladder stones (The American College of Gastroenteroly, 2008), the study of intestine and its complexity is a field of research that needs more attention for obesity approaching.

Altered intestinal permeability could be a reflex of obese dietary habits and microbiota (Cani et al, 2007), and animal models of obesity (ob/ob, db/db) suggest its relation to insulin sensitivity and fatty liver (Brun et al, 2006; Cani et al, 2009). The aim of this review was to discuss possible mechanisms underlying altered intestinal permeability in obesity and its link with obesity-related comorbidities.

2. Lactulose/Mannitol ratio: suitable for intestinal permeability assessment in obesity?

Once it is a non-invasive methodology, measurement of intestinal permeability, through orally ingested macromolecular probes and their quantification in the urine, is the most widely accepted method for evaluation of intestinal barrier integrity in humans (Farhadi et al, 2003a). Under normal conditions, molecules the size of disaccharides (lactulose) are restricted from moving across the villus tip, whereas smaller molecules (mannitol) can do so with relative freedom as the smaller channels are concentrated at the villi tips (Arrieta et al, 2006). Thus, smaller molecules such as mannitol are expected to be present in urine in higher proportion than bigger molecules like lactulose. The calculation of the lactulose:mannitol (L/M) ratio is considered a good marker of small intestinal permeation (Farhadi et al, 2003b) and is meant to circumvent confounding factors as inter-individual variation of gastric emptying, intestinal transit and transport, blood distribution and renal clearance (Martínez-Augustin, 1995).

Theoretically, an increase in the L/M ratio may be caused by a decrease in mannitol absorption and/or an increase in lactulose absorption. Decreased mannitol absorption can be the result of a diminished absorptive area, while an increased permeation of lactulose may be due to a facilitated diffusion of this sugar into the crypt region as a consequence of decreased villous height or tight junction loosening (Hulst et al, 1998). This ratio is of particular importance in diseases where the villosity is lesioned, because from a clinical perspective in these cases there is a marked reduction in mature small intestinal surface area, such as celiac disease, and consequently a substantial reduction in the fractional excretion of small probes such as mannitol (Arrieta et al, 2006), what would result in an increased ratio.

In the case of obesity, abnormal distribution of tight junctions proteins (Brun et al, 2006) and higher inflammatory tonus influenced by the microbiota (Cani et al, 2007)

could be pointed as possible mechanisms to support the belief that tight junction might be loosen in obesity, contributing to higher paracellular substances permeation, such as increased lactulose absorption. But the L/M ratio might not be the best marker of altered intestinal permeability because obese individuals might be absorbing proportionally higher quantities of mannitol, so that the increased excretion of lactulose does not appear with the calculation of the ratio. Two studies provide evidence to hold this hypothesis. Ferraris & Vinnakota (1995) showed in animal model that genetic obesity is associated with increased intestinal growth, which augments absorption of all types of nutrients. McRoberts et al (1990) showed in a cell culture model that the addition of insulin – which is a hormone usually increased in obese subjects (Kahn et al, 2006) - at the media to the serosal (basolateral membrane) induced a decline in transepithelial resistance while at the mucosal site (apical membrane) there was no significant effect, suggesting that the insulin-induced decline in transcellular resistance is receptor-mediated and that receptors are localized in the basolateral membrane. Increased mannitol flux was an observed effect paralled to this altered paracellular permeability.

Besides, D´Souza et al (2003) demonstrated that mannitol fluxes across Caco-2 cells cultured in high glucose media increased by 65% and it predominantly affected transepithelial transport of solutes permeating the cell barrier by paracellular and transcellular passive diffusion.The luminal content of glucose might be high in obese individuals due to the common pattern of high glycemic index foods consumption and added sugars (Ludwig et al, 1999; Jenkins et al, 2000; Wylie-Rosset et al, 2004; Drewnowski, 2007). Dietary glycemic index is positively associated with the HOMA index and the prevalence of metabolic syndrome (McKeown et al, 2004). The HOMA index and insulin values rise for insulin-resistant patients and insulin resistance is commonly associated with obesity (Keskin et al, 2005).

Thus, our hypothesis that L/M ratio might mask altered intestinal permeability, due to increased absorption of both sugar probes, deserves further investigation.

3. Possible causes of altered intestinal permeability in obesity

3.1. Dysbiosis

Dysbiosis can be characterized by an altered composition and/or distribution of the microbiota, and obesity has been characterized by both. The gut microbiota of obese mice and humans include fewer *Bacteroidetes* and correspondingly more *Firmicutes* than that of their lean counterparts (Ley et al, 2006; Dibaise et al, 2008). Furthermore, high prevalence of small intestinal bacterial overgrowth (SIBO) has been detected in severely obese patients (Sabaté et al, 2008). This also has been detected in diseases related to altered intestinal permeability (Parodi et al, 2009) supporting the principle that obesity is linked with an altered intestinal permeability.

The mechanism underlying the role of dysbiosis in the development of altered intestinal permeability is related to the altered immune responses due to disturbance of the partnership between the microbiota and the host immune system, ultimately leading to inflammatory disorders through cytokines secretion. The fact that the intestinal microbiota can exert both anti and pro-inflammatory effects (Round & Mazmanian, 2009), antibiotic therapy improves intestinal permeability (Cazzato et al, 2008) and that probiotic bacteria increases tight junctions resistance and reduces cellular permeability (Zareie et al, 2006) via modulation of cytokine production (Resta-Lenert & Barret, 2006) reinforce the importance of a balanced microbiota to a proper intestinal barrier function in obesity.

3.2. Nutritional deficiencies

The low intake or deficiencies of some nutrients have been reported in obesity: vitamin A (Ribot et al, 2001; Bonet et al, 2003; Zulet et al, 2008), zinc (Chen e Lin, 2000; Lee et al, 1998; Ozata, et al, 2002), vitamin D (Wortsman et al, 2000; Snijder, et al, 2005; Botella-Carretero et al, 2007) and calcium (Parikh & Yanovski, 2003; Schrager, 2005; Liu et al, 2006).

Retinoic acid cellular availability is regulated by the nutritional status of vitamin A and metabolic depletion of retinoic acid in cells were clearly related to dysfunctional epithelial barrier once it plays a role in the expression of genes related to tight junctions (Osanai et al, 2007). Zinc supplementation help to decrease lactulose excretion (Sturniolo et al, 2001; Chen et al, 2003), but the mechanisms are not established.

Vitamin D receptor plays a critical role in mucosal barrier homeostasis because of its ability to preserve junctional complexes integrity and stimulate epithelia renewal (Kong et al, 2008), and indirectly modulates the immune system (Deluca & Cantorna, 2001; Mathieu et al, 2004; van Etten & Mathieu, 2005). Vitamin D regulates calcium metabolism through its action on intestinal cells to increase calbidin expression and calcium absorption. Calbidin modulates the activity of calcium ATP-dependent pump located in the basolateral membrane of intestinal cells (Wood et al, 1998). An ATP depletion-repletion model for ischemia and reperfusion injury in kidney cells showed that lowering intracellular calcium during ATP depletion is associated with significant inhibition of the reestablishment of the permeability barrier following ATP repletion (Ye et al, 1999). This kind of mechanism should be investigated in intestinal cells.

4. Possible mechanisms that links altered intestinal permeability with metabolic consequences of obesity

4.1. Non-alcoholic fatty liver disease and insulin resistance

The liver is an important organ of metabolism and its function can be altered in obesity due to insulin resistance and endotoxins (as lipopolyssacharides, LPS) (Cani et al, 2007). In ob/ob mice the molecules of activation of the inflammatory cascade as TNF-α, IKKß, NFkB, JNK are typically increased in the liver with insulin resistance (Li et al, 2003).

Hepatic insulin resistance is caused by fat accumulation in the liver (Samuel et al, 2004). Although the gold standard for diagnosis of non-alcoholic fatty liver disease (NAFLD) is liver biopsy, ALT and AST levels is an indirect measure of liver alterations, as elevated levels and obesity would indicate a risk factor to predict advanced liver disease (McCullough, 2004). Normal levels of ALT, AST can be observed in patients with NAFLD even if there are histological alterations in the liver (Mofrad et al, 2003; Poniachik et al, 2006). The incubation of these patient´s blood with lipopolysaccharide (LPS) showed a response of elevated production of IL-1α and TNF-α, and a positive correlation between the degree of steatosis and HOMA index (Poniachik et al, 2006).

Bugianesi et al (2005) discuss that several mechanisms may account for fatty liver in insulin-resistant states, but factors responsible for the progression from simple fatty liver to non-alcoholic steatohepatitis (NASH) remain obscure. Furthermore, they

attribute to dysfunctional fat cells, with unbalanced cytokine activity originated from oxidative stress, the link between metabolic and liver disorders. In ob/ob mice, intestinal barrier dysfunction is related to higher LPS levels in portal circulation, to an increased hepatic macrophage infiltration and to the expression of oxidative stress markers (Cani et al, 2009). On the other hand, Farhadi et al (2008) suggest that there is a susceptibility to gut leakiness in obese subjects with NASH, which may be the cause for the higher endotoxaemia (higher levels of LPS) and consequently to the progression of NAFLD to NASH and advanced fibrosis. The reduction of bacterial compounds (LPS) by the use of antibiotics reduces liver inflammation and levels of ALT and AST (Bigorgne et al, 2008).

4.2. Low HDL levels

High density lipoprotein (HDL) concentration below 50mg/dL is a criterium considered for metabolic syndrome diagnosis, and usually obesity is related to lower concentration of HDL (Alberti et al, 2009; Singh et al, 2009). Insulin resistance theory provides mechanistic explanations to the observed tendency to lower HDL-cholesterol concentrations (Laws & Reaven, 1992; Razani et al, 2008) observed in the obese group. The prevalence of lower HDL concentrations increases from the lowest to the highest quintile categories of HOMA values (McKeown et al, 2004).

There is epidemiologic evidence that endotoxemia constitutes a strong risk factor of early atherogenesis in subjects with chronic or recurrent bacterial infections (Wiedermann et al, 1999). It has been suggested that chronic infections and inflammatory states can impair reverse cholesterol transport exerted by the HDL lipoprotein. During these states one of the consequences is lowering of the HDL concentration and of the proteins involved in the efflux of cholesterol from cells like macrophages. Endotoxin (LPS), which could be increased in portal blood in cases of increased intestinal permeability (Brun et al, 2006; Cani et al, 2007), has been shown to down-regulate the expression of proteins in the liver and macrophages involved in the first step of reverse cholesterol transport (efflux of cholesterol from the cells) (Khovidhunkit et al, 2003) and to facilitate foam cell formation, which is a proatherogenic factor (Baranova et al, 2002).

4.3. Hyperleptinemia

As the two main animal models (ob/ob and db/db) that link obesity with altered intestinal permeability are related to lack of leptin molecule or dysfunctional leptin

receptor we hypothesized that it might exists a positive correlation between body fat and altered intestinal permeability once leptin levels are related to adiposity (Considine et al, 1996).

There is the hypothesis that the maintenance of a chronic low-grade inflammatory state at metabolically relevant sites, such as the liver and adipose tissue, is involved in the progression of obesity and its associated comorbidities. This inflammatory state can be the result of adipose synthesis of tumor necrosis factor-alpha (TNF-α and leptin, once they can induce the production of IL-6, CRP and other acute-phase reactants (Bulló et al, 2003). The majority of obese individuals exhibit an elevated serum leptin levels commensurate with their adipose mass (Considine et al, 1996) what has been thought as a consequence of leptin resistance once higher levels of the hormone in obese patients fail to modulate appetite and to prevent or mitigate obesity (Myers et al, 2008).

Increased levels of leptin could be a protective mechanism to cope with TNF-α toxicity (Takahashi et al, 1999). In humans, it was found a positive association between leptin, soluble receptors of TNF-α and insulin levels (Mantzoros et al, 1997). The abnormal production of TNF-α is of metabolic significance once its blockage results in improved insulin resistance (Hotamisligil, 2003). The expression of TNF-α in the adipose tissue is 7.5 fold higher in obese individuals than in lean individuals, and this is inversely related to insulin sensitivity (Kern et, 2001) and may contribute to obesity-related hyperleptinemia (Kirchgessner et al, 1997).

Some studies provide evidence of the association between LPS, stimulation of the immune system and neuroendocrine system or leptin synthesis. Exogenous administration of leptin up-regulated phagocytosis in ob/ob animals and significantly increased the LPS-stimulated production of TNF, IL-6 and IL-2. Loffreda et al (1998) showed that enhanced macrophage synthesis of pro-inflammatory cytokines in response to LPS depends on leptin signals. LPS is a strong inducer of signaling pathways of inflammatory mediators like TNF-α secretion (Guha & Mackman, 2001). Primary adipocytes from endotoxin-sensitive and endotoxin-insentive mice were cultured in the presence of TNF-α or LPS and the results indicated that LPS induces leptin via a cytokine-dependent mechanism, and that TNF-α can act directly on adipocytes to stimulate leptin secretion (Finck et al, 1998). Animal models of infection show that injection of LPS, TNF and IL-1 increases the expression of leptin in adipose tissue and higher doses of LPS are related to higher expression of leptin with consequent food

intake suppression (Grunfeld, 1996). But it is the chronic infusion of LPS at very low dose that leads to metabolic changes related to obesity (Cani et al, 2007).

Thus, if an altered intestinal permeability could contribute to a low dose of LPS absorption, although leptin expression could be marginally induced, its increase wouldn´t be enough to produce impact on appetite modulation. But as leptin expression reflects an increase in inflammatory cytokines such as TNF-α, a subclinical inflammatory state could be chronically being developed together with the metabolic changes.

Another point of future investigations is the possible role of leptin in intestinal permeability, once leptin receptors have been identified in enterocytes and the lack of leptin or resistance to leptin action in this site affects lipid handling with the reduction of the apolipoprotein AIV in the jejunum (Morton et al, 1998) and also influences sugar absorption *in vivo*. In the study of Pearson *et al* (2001) infusion of leptin to rats that underwent 80% small bowel resection increased the absorption of galactose and GLUT-5 band intensity. Systemic infusion of leptin increases substrate absorption and mucosal mass in normal small intestine of rats acting as a growth factor (Alavi et al, 2002). But it was also found that luminal leptin inhibited sugar absorption and did not modify *in vivo* intestinal permeability determined with ^{14}C-mannitol (Iñigo et al, 2007).

Therefore it is suggested that the role of leptin on tight junction modulation and nutrient absorption should be investigated. If leptin increases due to LPS permeation (because of altered intestinal permeability, what would increase lactulose permeation and excretion) and if this increase act on intestinal mucosal to increase nutrient absorption (what would increase mannitol absorption and excretion), then the L/M ratio might not be adequate.

5. Future perspectives

Altered intestinal permeability precedes the development of increased adiposity, waist circumference and HOMA index, or the production of inflammatory factors from hypertrophic adipose tissue and insulin resistance could be the reason for the altered intestinal permeability? The last decade has been marked by the realization that obesity is linked with a state of chronic, low-grade, systemic inflammation (O´Rourke, 2009) originated from the white adipose tissue (WAT) as a result of chronic activation of

innate immune system. This results in an increased production and secretion of a wide range of inflammatory molecules including TNF-α and interleukin-6 (IL-6), which may exert local effects on WAT physiology but also systemic effects on other organs as well (Bastard et al, 2006).

Systemic inflammatory disorders can develop from an immune dysregulation with an inappropriate immune system over-activation as a result of a disproportionate penetration of luminal components (Farhadi et al, 2003b; Hollander, 2009). This information has clinical importance in the context of intestinal permeability in obesity. Firstly, because cytokine mediated changes in paracellular permeability, especially TNF-α and interleukins contribute to a multitude of pathological conditions (Capaldo & Nusrat, 2009). Secondly, a higher lactulose absorption and excretion reflects a dysregulated function of tight junctions and a leaky gut or a higher flux of molecules through the paracellular route (Farhadi et al, 2003b). Thirdly, it was found that the mucosal barrier function was significantly impaired in two different animal models of obesity, due to abnormal distribution of tight junctions proteins, favoring endotoxin leakage into the portal blood (Brun et al, 2006). Fourth, metabolic endotoxemia is defined as an subclinical increase in plasma LPS and chronic infusion of LPS at very low doses leads to metabolic changes such as increased glucose and insulin levels, weight gain (total, liver and adipose tissue), increased markers of inflammation in adipose tissue and hepatic triglyceride content (Cani et al, 2007). Finally, the infiltration of macrophages in the WAT is one of the causes of this higher production of pro-inflammatory molecules in obesity (Bastard et al, 2006), and we can hypothesize that this infiltration of immune cells in the adipose tissue could be the result of its enrichment with material derived from gut bacteria (LPS) once they are transported in the lymph through chylomicrons and inflammatory responses can be induced in the target tissues of this lipoprotein (Goshal et al, 2009).

In summary, studies support the hypothesis that dysbiosis and possible nutritional deficiencies are the main underlying explanatory cause for an altered intestinal permeability in obesity. This barrier function abnormality could probably be the cause or at least contributes to perpetuate obesity-related comorbidities. Protection of intestinal barrier as a prevention or therapeutic approach is an area of clinical and experimental research barely explored, but that harbors a promising field for future development of innovative interventions. To future studies design one should always bear in mind the link between intestinal permeability, microbiota, immune response,

cytokines, neuroendocrine response and obesity. The importance of gut health for weight control and prevention of obesity should be in deep debate among different research groups around the world.

6. References

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ARTIGO II:

Intestinal permeability parameters in obese patients are correlated with metabolic syndrome risk factors

ABSTRACT

In humans, if there is any association of altered intestinal permeability with adiposity and insulin resistance, therapies aimed at correcting abnormally increased intestinal permeability may play a role in the context of obesity. The aim of this study was to assess intestinal permeability in obese women and verify if there is any association with anthropometric measurements, body composition and biochemical variables. Twenty lean and twenty obese females participated in the study. Anthropometric measurements, body composition and blood pressure were assessed. Blood samples were collected to determine total cholesterol and lipoproteins, fasting glucose, fasting insulin, HOMA index, AST, ALT. A solution (120 mL) containing 6.25g lactulose and 3g mannitol was given to volunteers and their urine was collected over a period of 5h. The presence of these sugars in urine was measured by gas chromatography. The obese group presented lower HDL ($p<0.05$), higher fasting glucose, insulin, HOMA index and lactulose excretion than the lean group ($p<0.05$). Lactulose excretion presented positive correlation ($p<0.05$) with waist and abdominal circumference. Blood insulin and the HOMA index also increased with the increase in mannitol and lactulose excretion and L/M ratio ($p<0.05$). L/M ratio presented a negative correlation with HDL concentration ($p<0.05$). We showed that intestinal permeability parameters in obese women are positively correlated to anthropometric measurements and metabolic variables. Therapeutic interventions focused on the intestine health and modulation of intestinal permeability should be explored in the context of obesity.

Key words: intestinal permeability, insulin, HOMA, HDL, waist circumference

1. Introduction

Obesity is a worldwide epidemic problem (James et al, 2001; Ogden et al, 2007), and the metabolic syndrome is a common metabolic disorder resulting from this increased prevalence of obesity (Eckel et al, 2005). Insulin resistance, along with visceral adiposity, dyslipidemia and subclinical inflammatory state are the main features of this syndrome (Singh et al, 2009).

The intestinal barrier function has been viewed as an interface between health and disease (Farhadi et al, 2003a; Groschwitz & Hogan, 2009) and therapies toward correcting abnormally increased intestinal permeability may play a role in treating or preventing some diseases (Duerksen et al 2005; Arrieta et al, 2006; Vilela et al, 2007). Altered intestinal permeability has only been shown to affect obesity in animal models (Brun et al, 2006; Cani et al, 2009). Its reduction through the administration of prebiotic and changes in the microbiota improved systemic and hepatic inflammation, modulated gut peptides and adiposity (Cani et al, 2009) indicating that therapeutic approaches for improving intestinal permeability could positively impact on variables of metabolic syndrome.

Studies on intestinal permeability in obesity are justified by the fact that gastrointestinal bacteria can increase epithelial permeability (Fedwick et al, 2005) while small intestinal bacterial overgrowth (SIBO) was diagnosed in morbidly obese individuals and contributes to severe hepatic steatosis (Sabaté et al, 2008). On the other hand, the gut microbiota of obese individuals is statistically different from lean individuals (Tennyson & Friedman, 2008; Bäckhed et al, 2009; Turnbaugh et al, 2008) and the transference of obese microbiota to germ-free mice significantly increases total body fat (Turnbaugh et al, 2006). In humans, if there is any association of altered intestinal permeability with adiposity and insulin resistance, therapies aimed at correcting abnormally increased intestinal permeability may play a role in the context of obesity. Therefore, the aim of this study was to assess intestinal permeability in obese women and verify if there is any association with anthropometric measurements, body composition and biochemical variables.

2. Subjects and methods

2. 1. Subjects

Healthy women volunteers were recruited through written advertisements. Exclusion criteria were: younger than 18 years of age, pregnant or breast-feeding women, menopause, thyroid diseases, renal failure, cirrhosis, congestive heart failure, nephritic syndrome, diabetes, celiac disease, Crohn´s disease, irritable bowel syndrome, hepatitis, ulcers, use of vitamins/minerals supplements, use of non steroidal anti-inflammatory drugs, use of laxatives. According to a physical examination and a brief medical history, all subjects were in good health. Twenty lean females (BMI 19-24,99 kg/m^2) and twenty obese females (BMI > 30 kg/m^2) of similar age (mean age of the lean and obese group 28.5±7.6 *vs* 30.7 ± 6.5, p=0.33) participated in the study. None were taking any form of medication, except contraceptives pills. The study was approved by the ethical committee of Federal University of Viçosa and the participants provided written informed consent (protocol number 001/2010).

2.2. Study design

The subjects were evaluated at the Laboratory of Energetic Metabolism and Body Composition (LAMECC) at two occasions: the first to provide information about their health history and to receive all the recommendations prior to the next meeting. In the second meeting, subjects arrived in the morning at LAMECC after fasting for 10h and were asked to eliminate residual urine. All participants were weighted wearing light clothes, their body composition was analyzed by a tetra polar bioimpedance (BodySystems®, Washington, USA), blood pressure was assessed and blood samples were collected for future analyses. After that, a solution (120 mL) containing 6.25g lactulose (95%, Sigma-Aldrich®, Germany) and 3g mannitol (> 98%, Sigma-Aldrich®, Germany) was ingested, and urine was collected over a period of 5h. Two hours after the solution ingestion, the volunteers were offered a snack. At the end of this period, the whole volume of urine was measured and a aliquot of 50mL was taken and 0.01g of thimerosal (Labsynth®, Brazil) was added to prevent bacterial growth. The urine samples were stored at -20°C.

2.3. Intestinal permeability analysis

To quantify the sugars administered, urine samples were derivatized according to Farhadi et al. (2003b). Briefly, 200 µL of the urine samples were centrifuged for 20 minutes at 2250 rpm and 40µL of an internal standard solution was added (myo-inositol 20mg/mL (Fluka®, Switzerland) and phenyl-β-D-glucoside 20 mg/mL (Acrós Organics®, Belgium). The samples were then evaporated until dry at 70°C with continuous flux of nitrogen gas and ressuspended in 400µL of anhydrous pyridine (Sigma-Aldrich®, Germany) containing hydroxylamine (25 mg/mL, Sigma-Aldrich®, Germany). Next, it was heated to 70°C for one hour and centrifuged at 2250 rpm for 5 min. The supernatant (100 µL) was transferred to a vial and 200 µL of N-trimethylsilylimidazole (Acrós Organics®, Belgium) was added to react for 30 min at 70°C. From this derivative, 100 µL aliquot was transferred to an insert, and 1 µL was injected in a gas chromatograph (Shimadzu®, Japan) equipped with auto injector, flame ionization detector and capillary column (DB-5, 30m, 0,25mm x 0,25µm, J&W Scientific®, USA) for analysis. The parameters for sugar separation set in the gas chromatograph were adapted from Farhadi et al (2006) due to difficulties in lactulose detection using their original set conditions. Thus, the column temperature was set at 190°C for 5 minutes and then increased at a rate of 5°C/min for 12 minutes until reaching the final temperature of 250°C. This temperature was maintained for 15 minutes and the total run time was 32 minutes. The results were expressed as percentage of mannitol (%M) and lactulose (%L) excretion and as the Lactulose/Mannitol ratio (L/M).

2.4. Biochemical analysis

Blood samples were centrifuged at 3500 rpm for 15 min and the plasma was processed at the Laboratory of Clinical Analysis of the Health Division at Federal University of Viçosa. The biochemical assessment were hemogram (Coulter T-890/Beckman Coulter®, USA), total cholesterol and lipoproteins (enzymatic colorimetric method), aspartate (AST) and alanin (ALT) aminotransferases (kinetic colorimetric method), fasting plasma glucose (enzymatic colorimetric method of glucose-oxidase) (all the kits used were from Bioclin/Quibasa, Brazil) and insulin through quimioluminescence method using the Cobas Mira Plus-Roche automatic analyzer (Roche Diagnostics®). The LDL concentration was estimated by the Friedwald formula (Friedwald et al, 1972). Homeostasis model assessment (HOMA)

index were calculated as follow: fasting glucose (mmol/L) x fasting insulin (mU/L)/22,5 (Matthews et al, 1985; Oliveira et al, 2007).

2.5. Statistical Analysis

Statistical analyses were performed with the use of the software Sigma Plot for Windows version 11.0 (Systat® Software, Chicago, USA). To compare if all variables assessed differed between obese and lean individuals, student t-test (parametric) was used for those variables that passed in the normality and equal variance test while the Mann-Whitney test (non-parametric) was used for those that did not pass. The same tests and criteria were used to compare all the 40 volunteers distributed using as reference variables values below and above the median of lactulose and L/M ratio and the mean of mannitol excretion, below and above the threshold value for insulin resistance (HOMA index > 2.71) proposed by Geloneze et al (2006). The significance level was 5%.Throughout the manuscript, the data are expressed as means±SD and median (minimum-maximum). To measure the degree of correlation between intestinal permeability variables with other metric variables, the Pearson´s test was performed for mannitol excretion, which passed in the normality test, while the Spearman´s test was applied for lactulose excretion and L/M ratio once these variables did not pass in the normality test.

3. Results

3.1. Anthropometric and body composition variables and blood pressure

As shown in **table 1**, except for height that was similar in both groups, anthropometric and body composition variables and blood pressure differed between obese and lean groups as expected.

3.2. Biochemical analysis

The collection of blood sample from one volunteer of the lean group wasn´t possible because of her difficult venous access. All the variables related to hemogram did not differ between the groups (appendix I). Lipoprotein HDL was reduced in obese group, while the ratios of total cholesterol/HDL and LDL/HDL were increased ($p<0.05$). Fasting glucose, insulin and the HOMA index were also increased in the obese group ($p<0.05$) (**table 2**).

3.3. Intestinal permeability

The parameters percentage of lactulose and mannitol excretion and the ratio L/M are represented graphically in **figures 1, 2, 3** respectively. Mannitol excretion tended to be higher in the obese group, while lactulose excretion was higher in the obese group ($p<0.05$), but not sufficiently higher to significantly affect the L/M ratio (**Table 3**).

Lactulose excretion was significant and presented moderate positive correlation ($p<0.05$) with waist and abdominal circumference (**figure 4, 5**). The insulin concentration and HOMA index increased together with the increase in the percentage of mannitol (**figure 6, 9**) and lactulose (**figure 7, 10**) excretion and with the L/M ratio (**figure 8, 11**) ($p<0.05$), while HDL concentration (**figure 12**) presented a moderate inverse correlation with the L/M ratio ($p<0.05$) (**table 4**).

All 40 women were also analyzed dividing them by the median of percentage of lactulose excretion (appendix II). The group above the median presented higher body weight, BMI, waist and abdominal circumference, body fat weight and percentage, fasting insulin, HOMA index, % of mannitol excretion and L/M ratio ($p<0.05$). The use of the percentage of mannitol excretion mean (appendix III) as the criteria to divide all 40 women showed that those excreting a greater quantity of mannitol presented higher waist circumference and lactulose excretion ($p<0.05$).

Table 1- Anthropometric, body composition and blood pressure variables of obese and lean women

	Lean (n=20) Mean ±SD Median (min-max)	Obese (n=20) Mean±SD Median (min-max)	p value
Systolic blood pressure (mmHg)	104 ± 8.2 100 (90-120)	113 ± 10.3 120 (90-130)	0.005†
Dyastolic blood pressure (mmHg)	64 ± 8.8 60 (50-80)	74 ± 9.4 80 (60-90)	0.002†
Weight (kg)	55.2 ±5,2 54.6 (42.2-64.8)	88.06 ± 11.02 88.02 (74.6-118.1)	< 0.001†
Height (cm)	159.9 ± 5.6 159.5 (148.5-173.6)	158.5 ± 4.2 159 (150-168.2)	NS
BMI (kg/m^2)	21.5 ± 1.39 21.2 (19.22-23.9)	35.04 ± 3.98 34.4 (29.4-44.6)	< 0.001†
Waist circumference (cm)	69.57 ± 3.81 68.5 (65-77)	94.47 ± 8.16 94.5 (80.5-118)	<0.001†
Abdominal circumference (cm)	80.2 ± 4.33 80.5 (73-87.5)	110.02 ± 10.7 108 (96-138)	<0.001†
Hip (cm)	94.2 ± 3.5 93.5 (87-102)	117.17 ± 6.6 117.2 (106-133)	<0.001‡
Waist/hip ratio	0.738 ± 0.026 0.73 (0.707-0.794)	0.807 ± 0.061 0.79 (0.719-0.959)	<0.001‡
Body fat (%)	21.58 ± 3.52 22.6 (15.8-28.6)	37.48 ± 3.5 37.4 (29.4-43)	<0.001‡
Body fat weight (kg)	11.95 ± 2.6 11.8 (8.3-18.5)	33.23 ± 6.8 32.5 (21,9-50,8)	<0.001†
Lean mass (kg)	43.23 ± 3.76 43.5 (32.3-50.1)	54.7 ± 4.9 54.4 (47.3-67.3)	<0.001‡
Basal metabolic rate (Kcal)	1318.55 ± 115.6 1340 (983-1522)	1670.85 ± 152.73 1665 (1437-2047)	<0.001‡

†Mann-Whitney; ‡Student t test

BMI = Body Mass Index

Table 2 – Biochemical variables of obese and lean women

	Lean (n=19) Mean±SD Median (min-max)	Obese (n=20) Mean±SD Median (min-max)	p value
TC (mg/dL)	178.68 ± 32.9	169.5 ± 24.7	0.33‡
	178 (134-257)	166 (120-220)	
HDL (mg/dL)	55.4 ± 13.6	43.1 ± 9.35	0.001†
	52 (37-89)	42 (30-76)	
LDL (mg/dL)	107.32 ± 28.26	108.69 ± 25.18	0.87‡
	101.4 (65.2-172.8)	100.9 (68.6-168.4)	
TGL (mg/dL)	79.68 ± 31.12	88.75 ± 29.7	0.35‡
	73 (38-166)	85 (37-144)	
VLDL (mg/dL)	15.93 ± 6.22	17.76 ± 5.96	0.35‡
	14.6 (7.6-32.2)	17 (7.4-28.8)	
TC/HDL	3.37 ± 0.98	4.08 ± 1.07	0.016†
	3.09 (2.23-6.22)	3.84 (2.54-7.33)	
LDL/HDL	2.07 ± 0.87	2.65 ± 0.96	0.025†
	1.91 (1.07-4.67)	2.51 (1.32-5.61)	
Fasting glucose (mg/dL)	86.15 ± 5.49	89.8 ± 4.32	0.027‡
	86 (75-95)	89.5 (83-98)	
Fasting insulin (mcU/mL)	8.17 ± 2.59	14.8 ± 7.49	<0.001†
	8.1 (5-15.3)	11.4 (6.8-36.3)	
HOMA	1.74 ± 0.59	3.29 ± 1.71	<0.001†
	1.65 (0.96-3.47)	2.55 (1.39-8.15)	
AST (U/L)	19.21 ± 5.0	17.95 ± 3.88	0.47†
	18 (13-30)	17.5 (14-28)	
ALT (U/L)	14.78 ± 5.88	15.2 ± 5.73	0.82†
	13 (7-30)	14 (8-32)	

†Mann-Whitney; ‡Student t test

TC = Total cholesterol; HDL = High density lipoprotein; LDL = Low density lipoprotein; VLDL= Very low density lipoprotein; TC/HDL= total cholesterol/ high density lipoprotein; HOMA = Homeostasis Model Assessment; AST = Aspartate Aminotransferase; ALT= Alanine Aminotransferase

Table 3 – Variables of intestinal permeability of obese and lean women

	Lean (n=20) Mean±SD Median (min-max)	Obese (n=20) Mean±SD Median (min-max)	p value
% Mannitol excretion	17.32 ± 7.31 17.4 (1.12 -32.77)	21.86 ± 7.77 21.6 (7.72-39.89)	0.06‡
% Lactulose excretion	0.247 ± 0.087 0.23 (0.05-0.419)	0.418 ± 0.267 0.37 (0.057-1.069)	0.041†
L/M ratio	0.0144 ± 0.006 0.013 (0.007-0.034)	0.018 ± 0.008 0.015 (0.007-0.035)	0.13†

‡Student test, †Mann-Whitney test

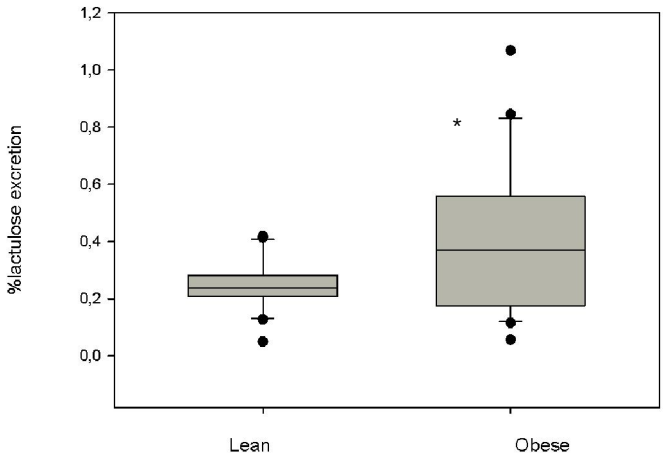

Figure 1: Percentage of lactulose excretion in the lean and obese groups. Obese group showed a higher lactulose excretion (p=0.041).

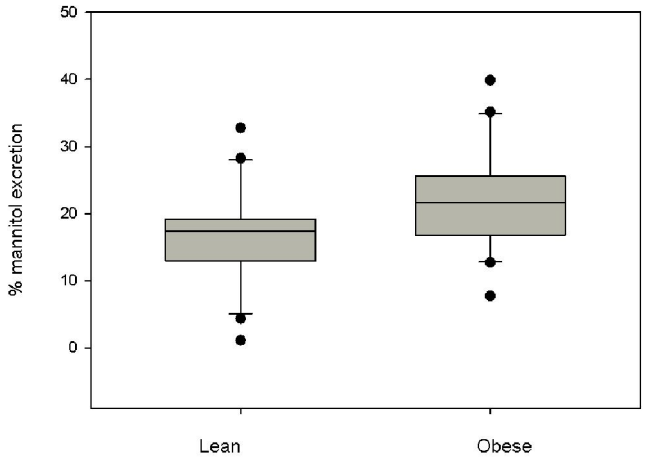

Figure 2: Percentage of mannitol excretion in the lean and obese groups. There was no difference between the groups (p = 0.06)

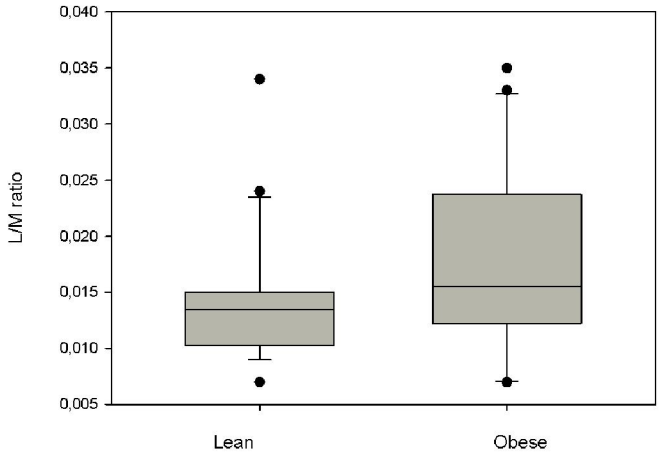

Figure 3: Lactulose/Mannitol ratio of lean and obese group. There was no difference between the groups (p=0.13).

On the other hand, when dividing all volunteers by the median of the L/M ratio (appendix IV) differences between the groups above and below the median of L/M ratio related to anthropometric and body composition variables were not observed, but women that presented L/M values above the median had lower HDL levels and higher values of TC/HDL ratio, LDL/HDL ratio, insulin, HOMA index and % of lactulose excretion ($p<0.05$).

The threshold value for HOMA index (>2.71) to characterize insulin resistance proposed by Geloneze et al (2006) for Brazilian population was also used to compare all

the variables. From this perspective, 25% of all volunteers (n=10) presented insulin resistance, being only one from the lean group and the others from the obese group. Comparing women with a HOMA index above and below 2.71 (appendix V) in the insulin resistant volunteers all anthropometric and body composition variables analyzed were higher ($p<0.05$). Interestingly, of all the comparisons performed, this was the only one in which it was observed higher values for leukocytes, lymphocytes and platelets ($p<0.05$) in the group above the cut-off point. They also presented higher mannitol and lactulose excretion percentages ($p<0.05$).

Table 4 – Correlation of intestinal permeability measurements with anthropometric, body composition and biochemical variables

Variables	r	p	r	p	r	p
	% M		% L		L/M	
Weight (kg)	0.18	0.26	0.30	0.05	0.28	0.07
BMI (kg/m^2)	0.18	0.25	0.24	0.12	0.25	0.10
Body fat (%)	0.19	0.24	0.27	0.08	0.24	0.13
Body fat (kg)	0.18	0.25	0.29	0.06	0.28	0.07
Waist circumference (cm)	0.22	0.16	0.32	0.04‡	0.28	0.07
Abdominal circumference (cm)	0.18	0.24	0.33	0.03‡	0.30	0.058
Waist/height ratio	0.22	0.16	0.25	0.10	0.18	0.25
HDL (mg/dL)	0.005	0.97	-0.27	0.08	-0.39	0.01‡
Fasting insulin (mcU/mL)	0.32	0.04†	0.46	0.002‡	0.35	0.029‡
HOMA	0.32	0.04†	0.47	0.002‡	0.39	0.014‡

†Pearson correlation test; ‡Sperman correlation test
%M = percentage of mannitol excretion; %L= percentage of lactulose excretion; L/M = Lactulose/mannitol

The mean value + 2 SD in the lean group for each of the intestinal permeability variables was used to verify how many volunteers would be above this value. Considering all 40 women, 10% (15% of the obese group and 5% of the lean group) were above the mannitol cut-off point (> 31.9% of excretion), 22.5% (45% of obese group and none of the lean group) for lactulose (> 0.0264% of excretion) and 12.5% (20% of obese group and 5% of lean) for the L/M ratio (>0.0264).

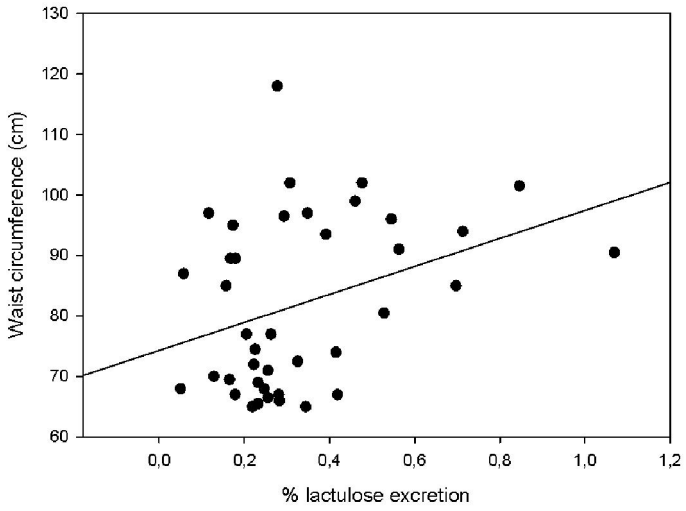

Figure 4: Correlation (Spearman test) between percentage of lactulose excretion and waist circumference (r=0.32, p=0.04).

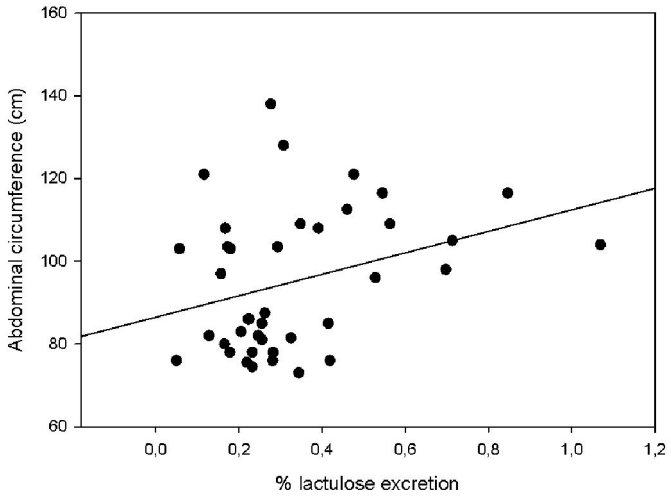

Figure 5: Correlation (Spearman test) between percentage of lactulose excretion and abdominal circumference (r=0.33, p=0.03).

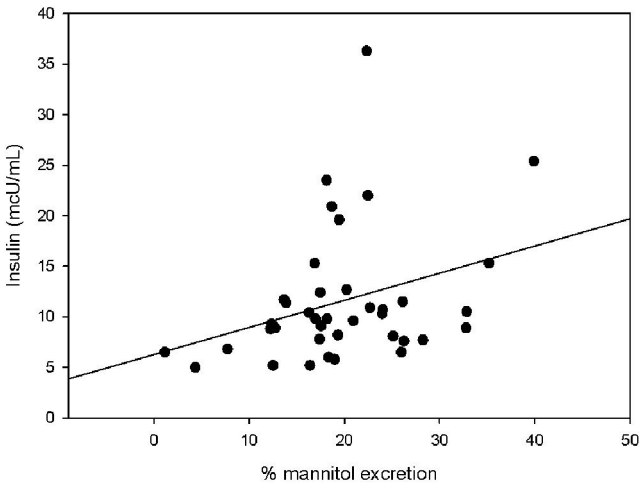

Figure 6: Correlation (Pearson test) between percentage of mannitol excretion and insulin (r=0.32, p=0.04)

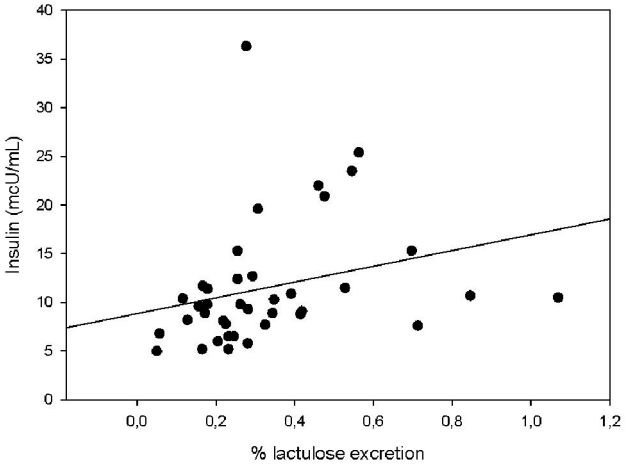

Figure 7: Correlation (Spearman test) between percentage of lactulose excretion and insulin (r=0.46, p=0.002)

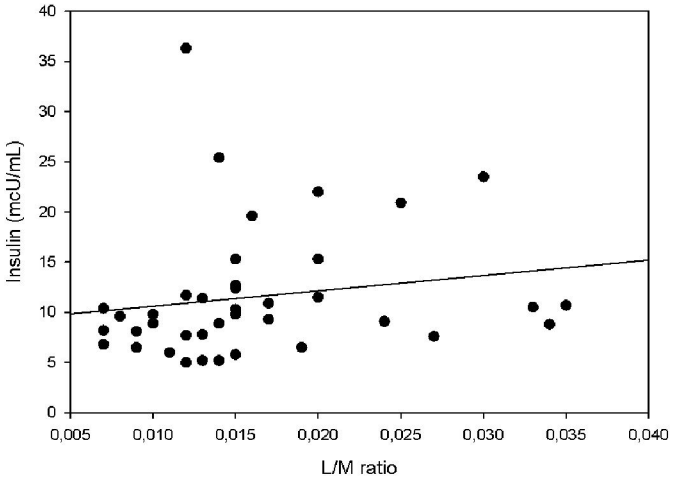

Figure 8: Correlation (Spearman test) between L/M ratio and insulin (r=0.35, p=0.029)

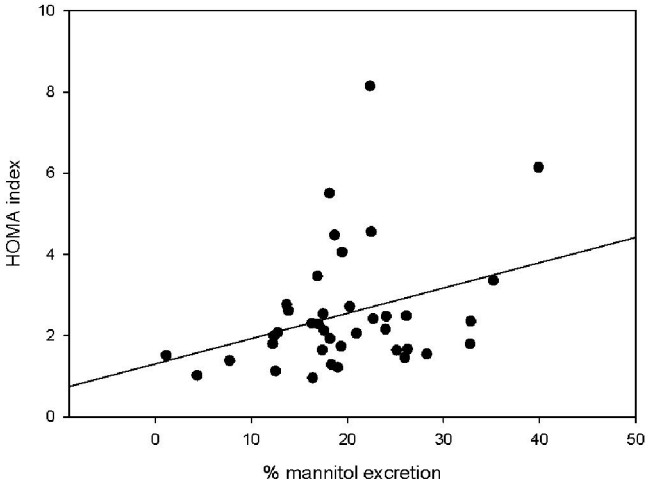

Figure 9: Correlation (Pearson test) between percentage of mannitol excretion and HOMA index (r=0.32, p=0.04)

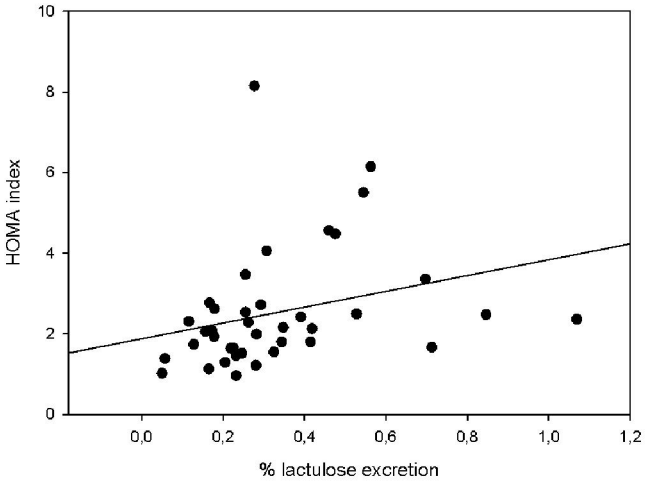

Figure 10: Correlation (Spearman test) between percentage of lactulose excretion and HOMA index (r=0.47, p=0.002)

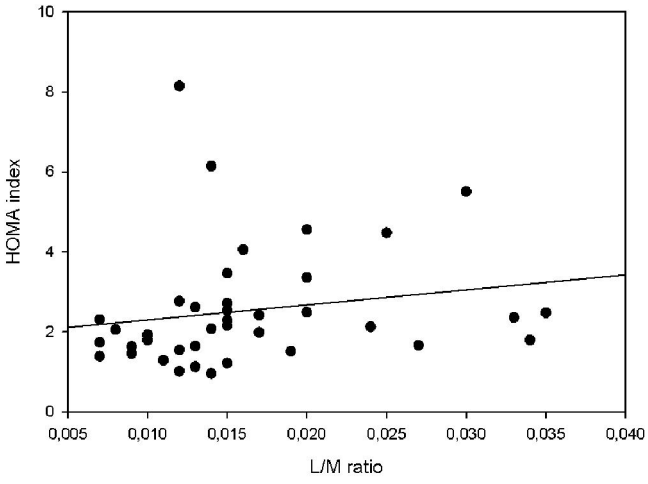

Figure 11: Correlation (Spearman test) between L/M ratio and HOMA index (r=0.39, p=0.014)

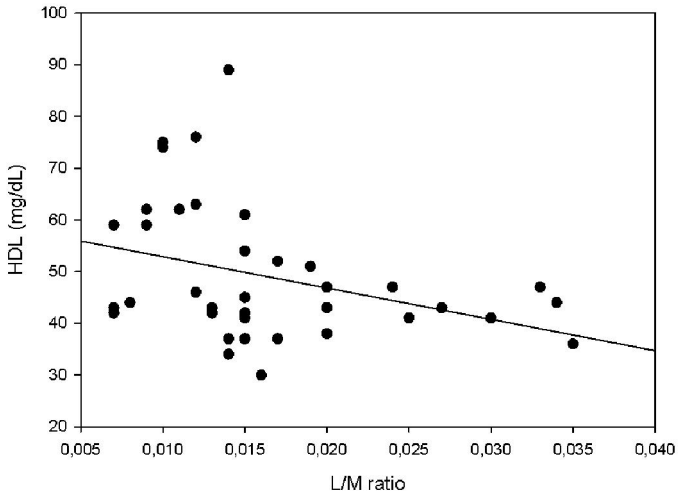

Figure 12: Correlation (Spearman test) between L/M ratio and HDL concentration (r= -39, p=0.01)

4. Discussion

The most critical factor in the emergence of metabolic diseases is obesity (Kahn et al, 2006). The interpretation of obesity as a disease from the perspective of excessive body fat (Bays et al, 2008; Waisbren et al, 2010) reinforce the term 'adiposopathy', which has been proposed to describe a pathogenic adipose tissue anatomically manifested by adipocyte hypertrophy, visceral adiposity and/or ectopic fat deposition, all of which could contribute to metabolic diseases (Bays et al, 2008). Obesity is one of the essential components included among some definitions proposed for metabolic syndrome, together with glucose intolerance, hypertension and dyslipidemia, (WHO, 1999; ATP III, 2001; Eckel et al, 2005). All of the screening variables used to identify this picture (Després & Lemieux, 2006) were evaluated in this study.

Waist circumference, which has been considered a useful preliminary tool for metabolic syndrome screening and prediction of body adiposity (Janssen et al, 2002; Alberti et al, 2009), was statistically higher in the obese group and their mean value was far beyond the predictive threshold value (≥ 80 cm) for abdominal obesity supported by IDF and AHA/NHLBI for South American ethnicity (Alberti et al, 2009). Blood pressure and fasting glucose, although within the limits considered normal, was already higher in the obese group, indicating that they might be progressing to a situation of

risk. Besides, HDL concentration in the obese group was significantly lower compared to the lean group and 90% of obese women were below the theorethical cut-off point for HDL concentration (< 50mg/dL) considered as a criterion for metabolic syndrome diagnosis (Alberti et al, 2009). The higher blood insulin and HOMA index, which are associated with higher abdominal fat accumulation, observed among obese volunteers signals to the presence of insulin resistance (Kahn et al, 2006). Insulin resistance theory provides mechanistic explanations to the observed tendency to higher blood pressure (Reaven et al, 1996), higher plasma glucose (Jellinger, 2007) and lower HDL-cholesterol concentrations (Laws & Reaven, 1992; Razani et al, 2008) observed in the obese group.

The upmost finding of this study was the higher lactulose excretion observed in the obese group and its positive correlation with waist and abdominal circumference, fasting insulin and HOMA index. The relation between altered intestinal permeability, insulin resistance and adiposity has been first suggested by experimental models (Cani et al, 2009; Brun et al, 2006), and we our results restate this hypothesis for obese women. It was shown that higher body weight, BMI, waist and abdominal circumference, body fat weight and percentage, fasting insulin and the HOMA index were found in women with higher lactulose excretion.

Although the calculation of the lactulose/mannitol ratio has been considered a good marker for small intestinal permeation (Farhadi et al, 2003a) the fact that it wasn´t observed statistical difference for L/M ratio among the groups does not invalidate the hypothesis that an altered intestinal permeability is one aspect of obesity. The questioning of L/M ratio as being the best marker for altered intestinal permeability in obesity should be raised. To argue with this hypothesis we point out to the fact that 1) volunteers with higher lactulose excretion percentages presented also higher mannitol excretion, and mannitol excretion tended ($p=0.06$) to be higher in obese women; 2) if we assume that obese individuals might be absorbing proportionally higher quantities of mannitol, the increased excretion of lactulose might not appear with the calculation of the ratio; 3) almost half of obese volunteers were above the mean+2SD of lactulose excretion (0.026%) calculated from lean individuals values. Our data also suggest that lactulose excretion and L/M ratio might be good indicators to be included to the list of criteria for metabolic syndrome diagnosis or management. Further studies should be designed to establish cut- off points of these probes excretion related to higher risk of metabolic alterations. Because higher lactulose absorption and excretion indicate a

dysregulated function of tight junctions and a leaky gut or a higher flux of molecules through the paracellular route (Farhadi et al, 2003a), future studies should also investigate the underlying causes of these altered paracellular permeability.

Some considerations should be pointed out, so that one might start drawing the possible mechanisms involved in many of the features of obesity: 1) higher lactulose excretion was positively correlated with HOMA index; 2) higher number of immune cells were observed when the volunteers were analyzed by the cut-off point for HOMA index; 3) obese microbiota is increased in relation to the *Firmicutes/Bacteroidetes* proportion (Bäckhed et al, 2009); 4) dysbiosis contributes to hepatic steatosis in obesity (Sabaté et al, 2008) and altered intestinal permeability has been shown in non-alcoholic fatty liver disease (Farhadi et al, 2008); 5) liver function's can be altered in obesity due to insulin resistance and endotoxins (LPS) (Cani et al, 2007); 6) LPS challenge is positively correlated to TNF-α and with the degree of steatosis and HOMA index (Poniachik et al, 2006); 7) dysregulated function of tight junctions could result in higher endotoxins or LPS uptake (Cani et al, 2007) and in the activation of the local or systemic immune system (Farhadi et al, 2003a; Hollander, 1999) with production of cytokines such as TNF-α (Guha & Mackman, 2001); 8) TNF-α can mediate changes in the paracellular permeability (Capaldo & Nusrat, 2009) and the chronic infusion of LPS at very low dose leads to metabolic changes related to obesity (Cani et al, 2007); 9) LPS induces leptin expression in adipose tissue via a cytokine-dependent (TNF-α) mechanism (Finck et al, 1998); 10) a positive association between leptin, TNF soluble receptors and insulin levels has been suggested (Mantzoros et al, 1997) and 11) higher TNF-α expression in the adipose tissue of obese individuals is inversely related to insulin sensitivity (Kern et, 2001) and may contribute to obesity-related hyperleptinemia (Kirchgessner et al, 1997). Thus, we can suggest that the interactions between gut microbiota, immune system, adipose tissue, liver and hormones are the main framework behind or underlying the altered intestinal permeability in obesity.

Future studies are needed to address the question of lactulose excretion as a better marker of altered intestinal permeability in obesity, which implies in higher paracellular absorption of substances, including bacterial material. So, further studies are also required to determine whether an altered intestinal permeability in obese individuals is associated with higher plasma endotoxin and leptin concentrations, immune system over activation and liver injuries.

As there is strong evidence that the detection, prevention and treatment of the underlying risk factors of the metabolic syndrome would be of importance to reduce cardiovascular disease incidence and mortality, as well as all-cause mortality (Galassi et al, 2006) our data suggest that therapeutic interventions focused in the intestine health and modulation of intestinal permeability should be explored in the context of obesity based on the findings that a positive correlation was found between higher lactulose excretion and anthropometric e metabolic alterations measurements.

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ARTIGO III:

Increased short-chain fatty acid in feces of obese patients correlates with metabolic syndrome risk factors

ABSTRACT

It has been postulated that the gut microbiota of obese individuals might harvest more energy from the diet and contribute for weight imbalance. Short-chain fatty acids (SCFA) are the main end products of bacterial fermentation and thus might play a role in obesity. The aim of this study was to assess the level of SCFA in feces of lean and obese individuals and verify if there is any association of possible increased production of SCFA with metabolic risk factors. Twenty lean and twenty obese females of similar age participated in the study. Anthropometric measurements, body composition, blood pressure and biochemical analyses were assessed. Feces were collected for short-chain fatty acid extraction and analysis by HPLC. Blood pressure and blood glucose, although within the normal limits, were higher in the obese group ($p<0.05$). Low-HDL concentration and high insulin and HOMA index values were observed in the obese group ($p<0.05$). The median values of butyric, propionic and acetic acid in the obese group was respectively 94.3%, 144.5% and 106.8% higher in comparison with the lean group ($p<0.05$). It was found significant correlation between SCFA and metabolic syndrome risk factors such as low HDL, increased waist circumference and HOMA index. In summary our results are in line with previous reports suggesting that increased SCFA production might play a considerable role in obesity once they are related to metabolic syndrome risk factors. Further studies are needed to confirm our findings.

Key words: short-chain fatty acids, insulin, HOMA, low HDL levels

1. Introduction

Obesity is one of the essential components, together with glucose intolerance, hypertension and dyslipidemia, included among some definitions proposed for metabolic syndrome (Eckel et al, 2005). Increased consumption and availability of high-energy foods in modern society are considered as one of the main causes of this global epidemic of obesity (Kopelman, 2000; Finkelstein et al, 2005). Recently, new evidences suggest a microbial role for obesity development (Bäckhed et al, 2009).

Microbial metabolism converts dietary macromolecules into absorbable molecules that can be used as an energetic substrate for the host. The higher energy extraction favors the anabolic pathways and inhibits fatty acid oxidation ultimately resulting in higher adiposity and lower glucose tolerance (Bäckhed et al 2007; Reinhardt et al, 2009). *Bacteroides thetaiotaomicron* are prominent members of the distal human gut microbiota and has an important contribution to carbohydrate metabolism (Zocco et al, 2007). Studies with germ-free animals colonized with this specie show fat mass gain and induction of monosaccharide transporters by the high activity of polysaccharides processing (Bäckhed et al, 2004) which is greater with the association with the *Methanobrevibacter smithii* specie (Samuel et al, 2008). These observations has led to the postulation that the gut microbiota of obese individual might harvest more energy from the diet and contribute to weight imbalance (Bäckhed et al, 2004; Turnbaugh et al, 2006).

Microbiota composition, together with substrate availability and intestinal transit time regulates the amount and proportions of SCFA in the gut (Macfarlane & Macfarlane, 2003). Short chain fatty acids (SCFA), which are organic fatty acids with 1-6 carbons (ex: acetate, propionate and butyrate), are the main end products arising from bacterial fermentation in the gut (Cook & Sellin, 1998; Zocco et al, 2007; Wong & Jenkins, 2007) mainly over carbohydrates resistant to digestion, carbohydrates that escape absorption in the small intestine and also proteins (Macfarlane & Macfarlane, 2003; Wong & Jenkins, 2007), and thus may play a role in obesity. In this context, the aim of this study was to assess the level of SCFA in feces of lean and obese individuals and verify if there is any association of possible increased production of SCFA with metabolic risk factors.

2. Subjects and methods

2.1. Subjects

The recruitment of female volunteers occurred using written announcements. Volunteers interested in participating in the study were screened by the phone. Inclusion criteria were: older than 18 years of age, not pregnant or breast-feeding, free of any liver, thyroid or gastrointestinal disease, not taking any kind of supplements or medications except oral contraceptive, being lean or obese.

The subjects were evaluated at the Laboratory of Energetic Metabolism and Body Composition (LAMECC) at two occasions: the first one to provide information about health history and to receive all the recommendations prior to the next meeting. In the second meeting, subjects arrived in the morning at LAMECC after fasting for 10h. They were weighted wearing light clothes, their body composition was analyzed by a tetra polar bioimpedance (BodySystems®, Washington, USA), blood pressure was assessed and blood samples were collected to biochemical analyses. Fresh feces samples were collected between these two occasions period or at the second encounter and were then immediately frozen at -20°C.

Twenty lean females (BMI 19-24,99 kg/m^2) and twenty obese females (BMI > 30 kg/m^2) of similar age (mean age of the lean and obese group 28.5±7.6 *vs* 30.7 ± 6.5, p=0.33) participated in the study. According to a physical examination and a brief medical history, all subjects were in good health. The study was approved by the ethical committee of Federal University of Viçosa and the participants provided written informed consent (protocol number 001/2010).

2.2. Biochemical analysis

Blood samples were centrifuged at 3500 rpm for 15 min and the plasma was processed at the Laboratory of Clinical Analysis of the Health Division at Federal University of Viçosa. The biochemical assessments were total cholesterol and lipoproteins (enzymatic colorimetric method), aspartate (AST) and alanin (ALT) aminotransferases (kinetic colorimetric method), fasting plasma glucose (enzymatic colorimetric method of glucose-oxidase) (all the kits used were from Bioclin/Quibasa, Brazil) and insulin through quimioluminecsence method using the Cobas Mira Plus-Roche automatic analyzer (Roche Diagnostics®). The LDL concentration was estimated by the Friedwald formula (Friedwald et al, 1972). Homeostasis model assessment

(HOMA) index were calculated as follow: fasting glucose (mmol/L) x fasting insulin (mU/L)/22,5 (Matthews et al, 1985; Oliveira et al, 2007).

2.3. Fecal short-chain fatty acids analysis

Short-chain fatty acids extraction was adapted from Smiricky-Tjardes et al (2003). Briefly, around 800 mg of fresh/wet feces were weighted and 1mL of m-phosphoric solution (25%) was added and homogenized. After 30 min rest at room temperature samples were centrifuged once at 13500 rpm during 30 min at 4°C and the supernatant were transferred to another vial. Another centrifugation and supernatant collection were processed and it was subsequently frozen at -20°C. A third centrifugation was performed before analysis. The short chain fatty acids - butyric, propionic and acetic - were measured by gas chromatography (model CG-17A, Shimadzu®, Japan) equipped with flame ionization detector and capillary Nukol column (30m x 0.25 mm, Supelco®). Nitrogen was used as the carrier gas and the flux in the column was 1.0 mL/min. The temperatures of the injector and detector were set at 220ºC and 250ºC, respectively. Initial column temperature was 100°C sustained for 5 min, rising at 10°C/min until reaching 185°C. Samples were injected (1 μL) through Hamilton® syringe (10μL) in split system 5. The total run time was 33.5 min. The data are expressed as mmol/L and represents the concentration of the fatty acids in the supernatant.

2.4. Statistical Analysis

Statistical analyses were performed with the use of the software Sigma Plot for Windows version 11.0 (Systat® Software, Chicago, USA). To compare if all variables assessed differed between the obese and lean groups student t-test was used for those variables that passed in the normality and equal variance test while Mann-Whitney was used for those that did not pass. Throughout the manuscript, the data are expressed as median (minimum-maximum). To measure the degree of correlation between each short chain fatty acid concentration with other metric variables Spearman´s correlation test was performed. ANOVA on ranks was used to verify if there were differences between the short-chain fatty acids concentrations of each group and Tukey test was used to all pairwise multiple comparison procedures. The level of significance considered in the tests was 5%.

3. Results

3.1. Subjects characteristics

As can be observed in table 1, although blood pressure parameters are below the threshold value for hypertension, they were higher for obese group ($p<0.05$). As expected, anthropometric and body composition variables were significantly higher for obese group ($p<0.05$). The biochemical variables are represented in table 2 and the main differences between the groups were regarding HDL, fasting glucose, insulin concentrations and the HOMA index.

3.2. Fecal short-chain fatty acids

It was observed an increased proportion ($p<0.05$) of all short-chain fatty acids in the obese group as shown in **figure 1**. The median values of butyric, propionic and acetic acid (table 3) in the obese group was respectively 94.3%, 144.5% and 106.8% higher in comparison with the lean group. Acetic acid was in higher proportions in both groups, but the proportion between them (acetic:propionic:butyric) considering median values was 2:1:1 in the lean group and 2:1.4:1 in the obese group.

Table 1- Anthropometric, body composition and blood pressure variables of lean and obese women

Variables	Lean (n=20) Median (min-max)	Obese (n=20) Median (min-max)	p value
Systolic BP (mmHg)	100 (90-120)	120 (90-130)	0.005†
Dyastolic BP (mmHg)	60 (50-80)	80 (60-90)	0.002†
Weight (kg)	54.6 (42.2-64.8)	88.02 (74.6-118.1)	< 0.001†
Height (cm)	159.5 (148.5-173.6)	159 (150-168.2)	NS
BMI (kg/m^2)	21.2 (19.22-23.9)	34.4 (29.4-44.6)	< 0.001†
Waist (cm)	68.5 (65-77)	94.5 (80.5-118)	<0.001†
Abdominal circumference (cm)	80.5 (73-87.5)	108 (96-138)	<0.001†
Body fat (%)	22.6 (15.8-28.6)	37.4 (29.4-43)	<0.001‡

BP= blood pressure; BMI = Body mass index, †Mann-Whitney; ‡Student t test

Table 2 – Biochemical variables of lean and obese women

	Lean (n=19)[a] Median (min-max)	Obese (n=20) Median (min-max)	p value
Total Cholesterol (mg/dL)	178 (134-257)	166 (120-220)	0.33‡
HDL (mg/dL)	52 (37-89)	42 (30-76)	0.001†
LDL (mg/dL)	101.4 (65.2-172.8)	100.9 (68.6-168.4)	0.87‡
TGL (mg/dL)	73 (38-166)	85 (37-144)	0.35‡
VLDL (mg/dL)	14.6 (7.6-32.2)	17 (7.4-28.8)	0.35‡
TC/HDL	3.09 (2.23-6.22)	3.84 (2.54-7.33)	0.016†
LDL/HDL	1.91 (1.07-4.67)	2.51 (1.32-5.61)	0.025†
Fasting glucose (mg/dL)	86 (75-95)	89.5 (83-98)	0.027‡
Fasting insulin (mcU/mL)	8.1 (5-15.3)	11.4 (6.8-36.3)	<0.001†
HOMA	1.65 (0.96-3.47)	2.55 (1.39-8.15)	<0.001†
AST (U/L)	18 (13-30)	17.5 (14-28)	0.47†
ALT (U/L)	13 (7-30)	14 (8-32)	0.82†

[a]One volunteer from this group had a difficult venous access and wasn´t included, †Mann-Whitney; ‡Student t test
TC = Total cholesterol; HDL = High density lipoprotein; LDL = Low density lipoprotein; VLDL= Very low density lipoprotein; TC/HDL= total cholesterol/ high density lipoprotein; HOMA = Homeostasis Model Assessment; AST = Aspartate Aminotransferase; ALT= Alanine Aminotransferase

Analyzing each group separately, acetic acid concentration were significantly higher ($p<0.05$) than butyric and propionic acids concentration in the lean group, but there was no difference between these two. On the other hand, in the obese group acetic acid was only higher than butyric acid, and there were not differences between acetic and propionic acid and propionic and butyric acid, indicating that the proportion of individual SCFA changed in favor of propionate in obese subjects.

Table 3 – Concentration (mmol/L) of short-chain fatty acids in feces supernatant from lean and obese group

SCFA (mmol/L)	Lean group (n=18)[a] Median (min-max)	Obese group (n=17)[a] Median (min-max)	p†
Butyric acid	4.25 (0.71-17.5)	8.26 (1.75-55.06)	0.02
Propionic acid	4.8 (1.9-12.1)	11.74 (2.87-355.6)	0.007
Acetic acid	8.46 (4.55-28.0)	17.5 (6.29-60.7)	0.007

[a]Not every volunteers brought a feces sample, † Mann-Whitney

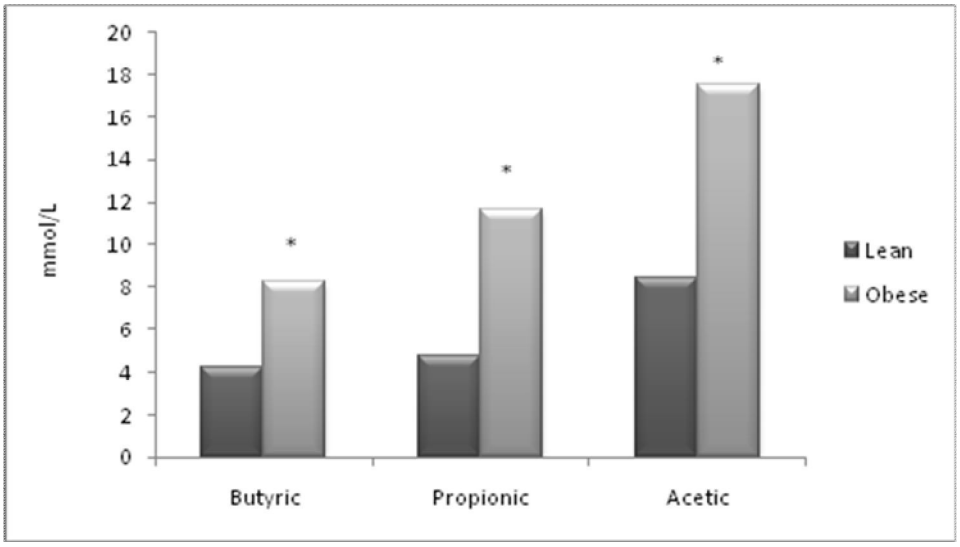

Fig 1 – Median values of SCFA in lean and obese group. * p<0.05 (Mann-Whitney)

Correlation tests were run for all variables analyzed in the study, but only the ones that presented a statistically significant correlation (p<0.05) were represented in table 4. As can be observed, variables related to metabolic syndrome diagnosis, such as waist circumference, blood pressure, HDL and insulin concentrations and HOMA index, were significantly correlated with short-chain fatty acids concentrations.

Table 4 – Correlation coefficient (r) from comparison of short-chain fatty acid concentration in feces supernatant with anthropometric, blood pressure and biochemical variables

Variables	**Butyric (r)**	**p‡**	**Propionic (r)**	**p‡**	**Acetic (r)**	**p‡**
Weight	0.35	0.044	0.44	0.009	0.47	0.005
BMI	0.34	0.05	0.46	0.007	0.49	0.003
Body fat	0.26	0.13	0.36	0.036	0.41	0.016
Waist circumference	0.36	0.036	0.43	0.012	0.50	0.002
Abdominal circumference	0.39	0.023	0.46	0.007	0.53	0.001
Systolic BP	0.39	0.0024	0.36	0.036	0.45	0.008
Diastolic BP	0.32	0.06	0.33	0.05	0.36	0.035
HDL	-0.38	0.03	-0.31	0.08	-0.57	0.0006
Insulin	0.46	0.007	0.53	0.001	0.60	0.0002
HOMA index	0.49	0.004	0.54	0.001	0.59	0.0003

BP = blood pressure, BMI = Body mass index, HOMA = Homeostasis Model Assessment,

‡Spearman correlation test

4. Discussion

The main finding of this investigation was in agreement with the study of Schwiertz and co-workers (2009) who found that the obese subjects show a higher concentration of acetate, propionate and butyrate compared to the lean group. They also found that the proportion of individual SCFA changed in favor of propionate in obese subjects. In general, fecal SCFA production is in the order acetate > propionate ≥ butyrate in an approximate molar ratio of 3:1:1, respectively (Topping & Cilfton, 2001). It is known that changes in concentration and proportion of individual SCFA are concurrent with changes in bacterial groups. Although we haven´t analyzed fecal microbiota, a group of investigators found that propionate producers belonging to the genera *Bacteroides* and *Prevotella* were numerous in overweight volunteers and there was significant correlation between propionate and body mass index (Schwiertz et al, 2009).

The colonization of mice with *Bacteroides thetaiotaomicron* and M*ethanobrevibacter smithii* increased fecal propionate and acetate concentration. These SCFA, especially propionate, are signaling molecules for the GPR41 receptor to increase host adiposity (Samuel et al, 2008) and leptin production (Xiong et al, 2004; Samuel et al, 2008). The loss of GPR41 is associated with reduced efficiency of energy harvest from the diet (Samuel et al, 2008), indicating that propionate interacting with GPR41 increases energy harvest. It was demonstrated in cell culture that acetate and propionate act on lipid accumulation and inhibition of lipolysis (Hong et al, 2005). This may explain the positive correlation found between propionic and acetic acid with weight, BMI, body fat percentage, waist and abdominal circumference found in this study.

Waist circumference has been considered a useful preliminary tool for metabolic syndrome screening and prediction of body adiposity (Janssen et al, 2002; Alberti et al, 2009) what indirectly also explain the direct correlation found for these SCFA with insulin and the HOMA index in the present study, once these are markers of metabolic syndrome related to fat accumulation (Carey et al, 1996; Arner, 2003). Insulin resistance theory provides mechanistic explanations to the observed tendency to higher blood pressure (Reaven et al, 1996), higher plasma glucose (Jellinger, 2007) and lower HDL-cholesterol concentrations (Laws & Reaven, 1992; Razani et al, 2008) observed in the obese group. The effect of SCFA on insulin resistance and these associated-metabolic disturbances are not well known. The SCFA are absorbed either by diffusion

or anion exchange, and sodium and water absorption is stimulated (Cook & Sellin, 1998), what may contribute to the positive relation between SCFA and blood pressure found.

Fasting glucose in the obese group, although within the normal limits, was higher than in the lean group. It has been demonstrated controversial effects of propionate over blood glucose concentration, reduction in rats (Boillot et al, 1995) and increase in humans (Wolever et al, 1991). Acetate may exert a glucose lowering effect since it promoted a small increase in plasma insulin and a decrease of plasma free fatty acids (FFA) in overweight individuals following lactulose ingestion. FFA reduction was related to a decrease in lipolysis (Ferchaud-Roucher et al, 2005), probably insulin-mediated, once this hormone stimulates lipoprotein lipase and the increase in the uptake of lipids from the circulation to the cell (Otarod & Goldberg, 2004). Although this action helps to reduce plasma FFA, it can favor higher body weight and fat percentage.

In the present study it was found a negative correlation between butyric and acetic acid with HDL. SCFA influence on HDL metabolism is not well established, and the other lipoproteins are usually more mentioned to be increased with acetate administration (Wolever et al, 1991; Wolever et al, 1996). Acetate and propionate have been proposed to have opposing effects in hyperlipidemia (Wong & Jenkins, 2007) and the effect may depend upon the relative proportions of acetate and propionate produced (Wolever et al, 1991). Weight loss diet with lower carbohydrate intake was associated with a higher increase in HDL concentration (Sacks et al, 2009). On the other hand, it was found that lower carbohydrate intake is associated with lower butyric acid production (Duncan et al, 2007). From these two different studies one may suppose a negative correlation between HDL and butyrate, but the exact mechanism still need to be established.

Butyric acid was the only short chain fatty acid that did not show a positive correlation with BMI and body fat. There is evidence that butyrate decreases β-adrenergic response in adipocytes inhibiting lipolysis (Krief et al, 1994) but also that it exerts protection against diet-induced obesity and insulin resistance (Gao et al, 2009). This last result differed from what was found in the present study, where a positive correlation between butyric acid and insulin concentration and the HOMA index was observed, and would partially explain the inverse relation to HDL concentration (Razani et al, 2008). From this perspective, it is possible a dual role for butyrate, maybe related to the concentration or the kind of bacteria present in the gut. Starch fermentation by

colonic bacteria favors the production of butyric acid, and that either gram-negative bacteria (*Bacteroides thetaiotaomicron*) or gram-positive (*Roseburia* and *Butyrivibrio*) compete effectively for the starch molecules (Ramsay et al, 2006). If butyric acid is increasing in response to gram-negative bacteria, there might result in an increase in the endotoxin levels (lipopolysaccharide) which can disturb the gut mucosal barrier (Courtois et al, 2003). It has been shown that an altered intestinal permeability would contribute to endotoxemia, a subclinical increase in plasma LPS, leading to metabolic changes related to obesity such as insulin resistance (Cani et al, 2007).

In summary our results are in line with previous reports suggesting that SCFA production might play a considerable role in obesity once they are related to metabolic syndrome risk factors such as low HDL, high waist circumference and the HOMA index. We have previously shown that the obese patients evaluated in this study also presented higher lactulose excretion, which didn´t present any correlation with short-chain fatty acids concentration in feces. On the other hand, as for SCFA, lactulose and mannitol excretion showed positive correlation with insulin and the HOMA index, but not with weight or body fat percentage. We hypothesized that the altered intestinal permeability would be the result of host interaction with gram-negative bacteria that increases in number due to the pattern of obese diet, and would favor permeation of endotoxins that contribute to insulin resistance development (Cani et al, 2009). Meanwhile, these bacteria would also be able to harvest higher energy from the diet through SCFA production, contributing directly to weight gain, adiposity and indirectly to insulin resistance. Further studies are needed to address the role of increased SCFA in feces of obese patients in the metabolic syndrome features.

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APPENDIX

APPENDIX I

Results of hemogram from lean and obese group

	Lean (n=19) Mean ± SD Median (min-max)	Obese (n=20) Mean ± SD Median (min-max)	p value
Erythrocytes	4.37 ± 0.344 4.39 (3.8-5.13)	4.39 ± 0.255 4.39 (3.93-4.94)	0.807
Hemoglobin	12.66 ± 1.03 12.8 (11.1-14.4)	12.6 ± 0.57 12.7 (11.7-13.5)	0.888
Hematocrit	38.6 ± 3.18 38.5 (34.1-45.5)	38.8 ± 1.65 39 (35.6-42.5)	0.603
MCV	88.35 ± 3.97 88.5 (79.1-97.7)	88.36 ± 4.29 88.7 (80.7-98.9)	0.996
MCH	28.9 ± 1.41 28.9 (25.6-31.8)	28.69 ± 1.71 28.5 (25.2-31.8)	0.647
MCHC	32.72 ± 0.67 32.7 (31.5-33.9)	32.45 ± 0.92 32.4 (30.9-34.5)	0.294
Leukocytes	5805.26 ± 1455.82 6000 (3200-8500)	6290 ± 1479.29 6450 (3500-8800)	0.309
Eosinophils	159.15 ± 109.33 128 (32-360)	151.2 ± 103.3 136 (0-360)	0.866
Lynphocytes	2007.5 ± 646,75 1938 (1152-3655)	2110.9 ± 522.75 2076.5 (1330-3344)	0.325
Monocytes	116.42 ± 86.77 114 (0-355)	118.4 ± 69.79 129 (0-255)	0.938
Platelets	209.57 ± 47.3 201 (135-331)	233.2 ± 67.29 217 (133-398)	0.376

MCV = mean corpuscular volume
MCH = mean corpuscular hemoglobin
MCHC= mean corpuscular hemoglobin concentration

APPENDIX II

Comparison of anthropometric, body composition and biochemical variables of women below (n=20) and above (n=20) the median of the percentage of lactulose excretion

	Women below % of lactulose excretion median Mean±SD Median (min-max)	Women above % of lactulose excretion median Mean±SD Median (min-max)	p value
Weight (kg)	64.4 ± 14.4	78.8 ± 19.9	0.013‡
	58.1 (49.7-92.6)	80.1 (42.4-118.1)	
Height (cm)	159.4 ± 5.2	159 ± 4.8	0.82
	159 (151.2-173.6)	159.1 (148.5-169.5)	
BMI	25.4 ± 5.7	31.2 ± 7.8	0.012‡
	22.6 (20.2-36.6)	32.6 (19.2-44.6)	
Waist circumference	76.15 ± 10.4	87.9± 15.02	0.007‡
(cm)	71.5 (65-97)	92.25 (65-118)	
Abdominal	88.5 ± 12.9	101.72 ± 18.55	0.013‡
circumference (cm)	84 (74.5-121)	104.5 (73-138)	
Hip (cm)	101.15 ± 10.8	110.22 ± 13.15	0.022‡
	96.2 (90.5-119)	113 (87-133)	
Body fat %	26.05±7.9	33.01 ± 8.2	0.01‡
	23.2 (15.8-38.9)	35.9 (16.4-43)	
Body fat (kg)	17.7 ± 9.5	27.4 ± 12.3	0.008‡
	13 (8.3-36)	29 (9-50.8)	
HOMA índex	1.89 ± 0.66	3.15 ± 1.8	0.008†
	1.74 (0.96-3.47)	2.45 (1.21-8.15)	
Fasting insulin	8.66 ± 2.78	14.3 ± 7.8	0.008†
	8.2 (5-15.3)	10.8 (5.8-36.3)	
% Mannitol	15.4 ± 6.02	23.7 ± 7.2	< 0.001‡
excretion	16.6 (1.12-25.9)	22.56 (12.2-39.8)	
% Lactulose	0.18 ± 0.06	0.47±0.21	< 0.001†
excretion	0.19 (0.05-0.26)	0.417 (0.27-1.06)	
L/M	0.011 ± 0.003	0.020 ± 0.007	<0.001†
	0.012 (0.007-0.019)	0.018 (0.01-0.035)	

‡Student t test; †Mann-Whitney test

APPENDIX III

Comparison of anthropometric, body composition and biochemical variables of women below (n=24) and above (n=16) the mean of the percentage of mannitol excretion

	Women below % of mannitol excretion mean Mean±SD Median (min-max)	Women above % of mannitol excretion mean Mean±SD Median (min-max)	p value
Weight (kg)	67.6 ± 17.6 58.9 (50.4-106.8)	77.6 ± 19.1 79.5 (42.4-118.1)	0.09‡
Height (cm)	159.7 ± 5.2 159 (151.2-173.6)	158.5 ± 4.7 159.3 (148.5-167.5)	0.47‡
BMI	26.6 ± 7.3 22.9 (19.6-43.7)	30.8 ± 7.0 32.1 (19.2-44.6)	0.08‡
Waist circumference (cm)	78.2 ± 12.7 73 (65.5-102)	87.6± 14.5 90.7 (65- 118)	0.036‡
Abdominal circumference (cm)	91.6 ± 16.6 85 (74.5-128)	100.2 ± 17 103.7 (73-138)	0.121‡
Hip (cm)	102.6 ± 12 96.2 (90.5-123)	110.2 ± 12.7 111.2 (87-133)	0.17‡
Body fat %	27.5± 8.6 23.8 (15.8-42.5)	32.5 ± 8.2 35.2 (15.8-43)	0.07‡
Body fat (kg)	19.9 ± 11.5 13.4 (8.4-45.4)	26.6 ± 11.7 27.9 (8.3-50.8)	0.20†
HOMA índex	2.26 ± 1.16 1.99 (0.96-5.5)	2.9 ± 1.8 2.39 (1.46-8.15)	0.17†
Fasting insulin	10.3 ± 5.08 9.1 (5-23.5)	13.3 ± 7.9 10.6 (6.5-36.3)	0.13†
% Mannitol excretion	14.7 ± 4.7 16.6 (1.12-19.4)	26.8 ± 5.5 25.5 (20.2-39.8)	< 0.001‡
% Lactulose excretion	0.24 ± 0.12 0.229 (0.05-0.54)	0.46±0.25 0.37 (0.15-1.06)	< 0.001†
L/M	0.011 ± 0.006 0.014 (0.007-0.034)	0.017 ± 0.008 0.015 (0.008-0.035)	0.589†

‡Student t test; †Mann-Whitney test

APPENDIX IV

Comparison of anthropometric, body composition and biochemical variables of women below (n=20) and above (n=20) the median of L/M ratio

	Women below L/M median Mean±SD Median (min-max)	Women above L/M median Mean±SD Median (min-max)	p value
Weight (kg)	68.4 ± 19.6	74.5 ± 17.7	0.213†
	61.4 (42.4-118.1)	79.4 (50.4-106.8)	
Height (cm)	158.8 ± 0.06	159.6 ± 3.7	0.35†
	158.5 (148.5-173.6)	160 (150-169.5)	
BMI	27.1 ± 7.3	29.4 ± 7.5	0.33†
	23.8 (19.2-44.6)	31.6 (19.6-43.7)	
Waist circumference	79.7 ± 14.3	84.09± 13.8	0.21†
(cm)	74.5 (65-118)	85 (66-102)	
Abdominal	92.1 ± 18.1	97.8 ± 16.07	0.13†
circumference (cm)	83 (73-138)	104.5 (73-138)	
Hip (cm)	103.2 ± 12.3	107.9 ± 13	0.30 †
	99 (87-123)	110.5 (90.5-133)	
Body fat %	28.2± 8.6	30.7 ± 8.9	0.45†
	24.5 (15.8-43)	34.3 (15.8-42.5)	
Body fat (kg)	20.7 ± 12.1	24.2 ± 11.7	0.32†
	14.7 (8.3-50.8)	27 (8.4-45.4)	
HOMA index	2.3 ± 1.8	2.76 ± 1.13	0.016†
	1.74 (0.96-8.15)	2.45 (1.21-5.5)	
Fasting insulin	10.4 ± 7.67	12.6 ± 5.18	0.022†
	8.2 (5-36.3)	10.8 (5.8-23.5)	
HDL	54.6± 15.6	43.8 ± 7.09	0.036†
	46 (34-89)	43 (30-61)	
TC/HDL	3.34 ±0.79	4.1 ±1.19	0.025†
	3.35 (2.23-5.4)	4.04 (2.23-7.33)	
LDL/HDL	2.02 ± 0.72	2.69±1.04	0.027†
	1.95(1.08-3.9)	(1.07-5.6)	
% Mannitol excretion	19.2 ± 8.5	19.8 ± 7.2	0.799‡
	18.1 (4.34-39.8)	18.9 (1.1-35.1)	
% Lactulose excretion	0.21 ± 0.11	0.44±0.22	< 0.001†
	0.179 (0.05-0.56)	0.39 (0.22-1.06)	
L/M	0.019 ± 0.002	0.021 ± 0.006	<0.001†
	0.012 (0.007-0.014)	0.019 (0.015-0.035)	

‡Student t test; †Mann-Whitney test

APPENDIX V

Comparison of anthropometric, body composition and biochemical variables of women without (n=30) and with (n=10) insulin resistance (HOMA index >2.71)

	Women without insulin resistance Mean ±SD Median (min-max)	Women with insulin resistance Mean ±SD Median (min-max)	p value
Weight (kg)	65.7 ± 15.5 59.5 (42.4-97.2)	89.35 ± 16.4 91.5 (58.9-118.1)	0.001†
Height (cm)			
BMI	25.9 ± 6.1 22.6 (19.2-37.4)	35.4 ± 6.3 36 (22.9-44.6)	0.001†
Waist circumference (cm)	77.7 ± 11.9 73.2 (65-101.5)	95 ± 12.3 96.2 (71-118)	0.001†
Abdominal circumference (cm)	89.5 ± 13.9 84 (73-121)	111.9 ± 15.11 110.7 (85-138)	< 0.001†
Hip (cm)			
Body fat %	27 ± 7.9 24.5 (15.8-41.3)	37.1 ± 6.6 37.4 (19.5-43)	< 0.001‡
Body fat (kg)	18.8 ± 9.8 14.1 (8.3-40.1)	33.9 ± 10.6 34.3 (11.5-50.8)	0.001†
Leukocytes	5617.2 ± 1348.5 5700 (3200-8500)	7320 ± 1029.3 7100 (5900-8800)	< 0.001‡
Eosinophils	153.7 ± 106.9 159 (0-360)	158.8 ± 104.4 129 (59-348)	0.84
Linfocytes	1957.9 ± 552.6 1949 (1152-3655)	2402.6 ±569.8 2211 (1820-3344)	0.036†
Monocytes	117.5 ± 80.8 114 (0-355)	117.2 ± 70.8 129 (0-213)	0.99
Segmented	3377.9 ± 1009.6	4669.9 ± 724.05	< 0.001‡

	3420 (1887-5382)	4611 (3658-6177)	
Platelets	205.5 ± 44.8	268.4 ± 71.6	0.008[†]
	200 (133-331)	251 (190-398)	
HOMA index	1.85 ± 0.48	4.5 ± 1.69	< 0.001[†]
	1.8 (0.96-2.6)	4.27 (2.7-8.15)	
Fasting insulin	8.57 ± 2.07	20.2 ± 7.2	<0.001[†]
	8.9 (5-12.4)	20.2 (11.7-36.3)	
% Mannitol excretion	18.03 ± 6.99	24.27 ± 8.58	0.026[‡]
	17.5 (1.12-32.8)	21.27 (13.65-39.89)	
% Lactulose excretion	0.306 ± 0.225	0.413 ± 0.16	0.026[†]
	0.239 (0.05-1.069)	0.402 (0.167-0.697)	
L/M	0.0158 ± 0.007	0.0174 ± 0.006	0.36
	0.0145 (0.007-0.035)	0.0155 (0.01-0.03)	