Antimicrobial emulsifier – glycerol monolaurate induces metabolic syndrome, gut microbiota dysbiosis and systemic low-grade inflammation in low-fat diet fed mice

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Abbreviations: GML, glycerol monolaurate; FPG, fasting plasma glucose; H&E, haematoxylin and eosin; HOMA-IR, homeostasis model assessment-insulin resistance index; PCoA, principal coordinate analysis; QIIME, Quantitative Insights into Microbial Ecology software; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.

Keywords: glycerol monolaurate, metabolic syndrome, gut microbiota, lipopolysaccharide, systemic low-grade inflammation

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Abstract

Scope: Glycerol monolaurate (GML) was widely consumed worldwide in food industry, and considered as safe, yet for chronic diseases, supporting scientific data remain sparse. This study was to investigate whether dietary GML induces metabolic syndrome, gut microbiota dysbiosis and systemic low-grade inflammation.

Methods and results: GML-induced occurrence of metabolic syndrome, gut microbiota alterations and systemic low-grade inflammation were investigated. Our results demonstrated that GML induced metabolic syndrome by significantly increasing the body weight, weight gain, food intake, body fat, fat droplet size and percentage of epididymal fat, serum triglycerides (TG), LDL and atherogenic index, and decreasing the body muscle ratio, liver weight and HDL, compared to the CON group. Meanwhile, GML significantly changed the β-diversity and composition of gut microbiota and up-regulated the circulating levels of serum LPS, IL-1β, IL-6 and TNF-α. Importantly, GML significantly decreased Akkermansia muciniphila and Lupinus luteus, and increased Bacteroides acidifaciens, Escherichia coli and the microbial DNA abundance of the 10 predicated metabolism pathways involved in carbohydrate, amino acid and lipid metabolism.

Conclusion: Our results indicate that the relatively low-dose GML consumption promotes metabolic syndrome, gut microbiota dysbiosis and systemic low-grade inflammation, thereby calling for a reassessment of GML usage.
1 Introduction

Metabolic syndrome is associated with an increased risk of obesity, type 2 diabetes and cardiovascular disease (CVD) [1, 2]. After an initial attempt by the WHO to define metabolic syndrome on the basis of certain criteria that included an insulin resistance marker, the NCEP–ATP III proposed simple screening tools and cut-off values to help identify patients who are likely to have features of metabolic syndrome. The five screening components used to identify those with metabolic syndrome are fasting plasma glucose, abdominal obesity, circulating levels of triacylglycerols, high-density lipoprotein (HDL)-cholesterol and blood pressure [3]. A meta-analysis of the prospective studies that have used these criteria has shown that the presence of metabolic syndrome increases the risk of obesity, type 2 diabetes and CVD [4].

Glycerol monolaurate (GML), a naturally occurring glycerol monoester of lauric acid enriched in coconut oil, is approved as a generally safe natural food emulsifier by the US Food and Drug Administration. The dosage of GML in food products is not limited and its additive amount in food and health care products ranges from 10 mg·kg\(^{-1}\) to 2000 mg·kg\(^{-1}\) (21 CFR GRAS 182.4505). In addition to its emulsification properties, GML also suppresses the growth and virulence of numerous bacteria, fungi, and enveloped viruses \textit{in vitro} [5-7]. For these reasons, GML are increasingly introduced into commonly consumed foods such as meat products, cereals and soft beverage [8-10]. Thus, GML is an effective antimicrobial-emulsifier that regularly consumed by the general public through its extensive commercial uses. Recently, growing evidence has indicated that the broad use of emulsifying agents might be contributing to an increased societal incidence of obesity/metabolic syndrome and other chronic inflammatory diseases [11]. Furthermore, Muthuramu \textit{et al} demonstrated that
coconut oil containing rich GML aggravated pressure overload-induced cardiomyopathy [12]. However, it remained unknown whether GML produces any effect on metabolic syndrome.

GML passes through the gastrointestinal tract with relative stabilization and long residence time [5, 13] and thus directly encounter the gut microbiota, which has great impacts on host health and physiology, especially in metabolism and immune development [11]. It is now widely accepted that the numerous symbiotic gut bacteria exert a sustained influence on the host’s immune system and metabolism through interactions of microbial cell components and gene products (e.g. lipopolysaccharides (LPS), peptidoglycan and flagellin) [14]. Recent studies have demonstrated that gut microbiota dysbiosis is a critical factor in the development of obesity and metabolic syndrome, possibly due to the increased energy harvest, production of toxic bacterial metabolites and greater intestinal permeability leading to elevated levels of LPS in systemic circulation, and consequently a low grade systemic inflammatory state [11, 15, 16]. Thus, a better understanding of the effects of dietary GML on modulating gut microbiota would be important for evaluating the safety of its use in consumed foods. However, it is completely unknown whether dietary GML induces gut microbiota dysbiosis and systemic low-grade inflammation.

In our unpublished studies, we found that dietary supplementation with 150 mg·kg⁻¹ GML significantly increased body weight and thickness of abdominal adipose tissue in pigs. To directly answer above questions, we studied the relatively low-dose GML-mediated modulation of community structure, composition and functional genes of gut microbiota, and the resultant effects on host metabolic syndrome and systemic low-grade inflammatory in mice.
2 Materials and methods

2.1 Animals

Male C57BL/6 mice were purchased from SLAC Laboratory Animal (Shanghai, China) and housed in the Zhejiang University animal facility in a specific pathogen free level room in hard top cages with two or three mice per cage. Mice were maintained at 22–24 °C in a 12-h light/dark cycle, and allowed ad libitum access to food and water. All procedures were conducted in accordance with institutional guidelines and were approved by the Animal Ethical Committee of Zhejiang University (reference protocol number ZJU-BEFS-2015012).

2.2 GML treatment

Mice were randomly allocated into two groups (n = 10 per group): (1) the basal Purina rodent chow diet no. P1100F (SLACOM Inc, Shanghai) as the control group (CON); (2) the basal diet supplemented with 150 mg·kg\(^{-1}\) GML as the treatment group (GML group) (Supplementary table 1). Each treatment has 4 cages (3 or 2 mice per cage). Body weights were individually measured every week and expressed as a percentage compared to the initial body weight (0 day) defined as 100%. Fresh faeces were collected every week for subsequent analysis. After 8 weeks of GML treatment, mice were fasted for 12 h, and blood was collected by retrobulbar intraorbital capillary plexus. Mice were then euthanized by i.p. injection of pentobarbital (200 mg·kg\(^{-1}\)), and liver weight and epididymal adipose weight were measured. Organs were collected for subsequent analysis. Incorporation of GML into the basic diet (Purina Rodent Chow diet no. P1100F) and maintaining total energy balance between diets by replacing isoenergetic fat with GML were performed by SLACOM Inc. (Shanghai, China).
2.3 DNA extraction and PCR

DNA extractions from the 8-week fecal samples (n = 10 per group) were performed using the Qiagen QIAmp DNA stool extraction kit (Qiagen) following the manufacturer’s protocol. DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μl using sterile water.

PCR amplification of 16S rRNA V4 regions were conducted by using the 515F/806R bacterial primer with the barcode (515F: 5’-GTGCCAGCMGCCGCGGTAA-3’ and 806R: 5’-GGACTACHVGGGTWTCTAAT-3’) [17]. PCR reactions were carried out in 30 μL reactions with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs); 0.2 μM of forward and reverse primers, and 10 ng templates DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 60 s, finally 72 °C for 5 min.

2.4 16S rRNA high-throughput sequence analysis

PCR products were mixed in equidensity ratios. Then, the mixture were purified with GeneJET Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer’s recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina MiSeq platform and 250 bp paired-end reads were generated [18]. Paired-end reads from the original DNA fragments were merged using FLASH and were assigned to each sample according to the unique barcodes. Sequence analysis was performed by UPARSE software package using the UPARSE-OTU and UPARSE-OTUref algorithms. Sequences with ≥ 97% similarities were assigned to the
same OTUs. A representative sequence was picked for each OTU and the Greengenes reference database was used to annotate taxonomic information for each representative sequence. In order to compare diversity, the OTU table was rarified and three metrics was calculated: Chao1, Observed species and Shannon index. The OTU absolute abundance table was extracted from the pipeline and converted to relative abundances by normalizing to total OTU clustering for analyzing the composition of gut microbiota and predicating the variation of functional genes by Quantitative Insights into Microbial Ecology software (QIIME) and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt).

2.5 Serum lipid parameters and fasting blood glucose testing

Total serum cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), fasting plasma glucose (FPG) and insulin (FINS) were determined using a kit from Nanjin Jiangcheng Bioengineering Institute (Jiangshu, China), following the manufacturer’s instructions. Concentrations of low-density lipoprotein cholesterol (LDL-C) were calculated using the Friedewald equation: LDL cholesterol = total cholesterol – HDL cholesterol – TG/2.2. Atherogenic index = (TC– HDL-C)/HDL-C and homeostasis model assessment-insulin resistance index (HOMA-IR) = FPG* FINS/22.5 [19].

2.6 Body composition analysis

Body fat and muscle percentage were determined with the nuclear magnetic resonance system using a Body Composition Analyzer MesoQMR06-100H (Suzhou Niumag Analytical Instrument Corporation, China), as described previously [20].
2.7 Liver and epididymal adipose tissue histopathology

Mouse liver and epididymal adipose tissue samples (n=5) were fixed in 10% buffered formalin for 24 h at room temperature and then embedded in paraffin, respectively. Tissues (five sections per mouse) were sectioned at 5 μm thickness and stained with haematoxylin and eosin (H&E) using standard protocols, and the number and size of stained fat droplets in epididymal adipose tissue were analysed by the Image J software (National Institutes of Health, USA) [15].

2.8 Measurement of systemic cytokine levels and serum LPS concentration

Enzyme-linked immunosorbent assay (ELISA) kits were used to determine serum levels of IL-1β, IL-6, TNF-α and IL-10 (eBioscience), according to the manufacturers’ instructions. Serum LPS concentrations were measured with a ToxinSensor Chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript, Piscataway, NJ), following the manufacturer’s instructions. Before measuring LPS concentrations, the serum were heated for 10 min at 70 °C to minimize inhibition or enhancement by contaminating proteins. LAL reagents were added to serum and incubated at 37 °C for 45 min, and the absorbance was read at 545 nm. All samples were validated for recovery and internal coefficient variation using known amounts of LPS.

2.9 Statistical analysis

The data of results were collected individually, calculated for each treatment, and expressed as mean ± standard error (SE). Ten mice were used in each treatment and each mouse was considered as experimental unit. For food intake, four cages were used in each treatment and each cage was considered as experimental unit. Statistical differences between more than two time-points were evaluated by one-way analysis of
variance (ANOVA) with Tukey’s multiple comparison post-tests. Data between two treatments were
determined using the unpaired two-tailed T-test in GraphPad Prism version 5 (GraphPad Software, La Jolla,
CA). Data from high-throughput sequence between two treatments were analyzed with T-test in QIIME-1.8.0
and R 3.1.0. Data were checked for heterogeneous variance with the Brown-Forsythe tests or the F test.
P-value < 0.05 was considered significant and 0.05 < P-value ≤ 0.10 were discussed as tendencies.

3 Results

3.1 GML induces metabolic syndrome

In order to investigate whether GML induced metabolic syndrome, a relatively low concentration of GML was
utilized to feed mice for 8 weeks. Markedly, the body weight, weight gain, food intake, body fat percentage
and epididymal fat in the GML group were significantly increased (P < 0.001, P < 0.001, P < 0.001, P = 0.037 and
P = 0.047, respectively), compared to those of the CON group (Fig. 1a). Moreover, the body muscle percentage
and liver weight were significantly decreased in the GML group (P < 0.001 and P = 0.045, respectively),
compared to the CON group. Interestingly, H&E histopathology demonstrated that the size of stained fat
droplets of epididymal adipose tissue in the GML group were significantly larger (P < 0.001) than that of the
CON group, but not for the stained fat droplets of liver (Fig. 1b and c). Meanwhile, the frequency of 500 ~ 1000
μm² of stained fat droplets of epididymal adipose tissue in the GML group were significantly decreased (P <
0.01), and the frequency of more than 1500 μm² of fat droplets in the GML group tended to increase,
compared to the CON group (Fig. 1b). Importantly, the serum TG, LDL and atherogenic index in the GML group
were significantly higher, and serum HDL in the GML group was significantly lower than those in the CON
group (Fig. 2a and b). However, the serum TC, fasting plasma glucose, insulin and HOMA-IR were moderately
increased, and no significant differences were observed between the GML and CON groups.
3.2 GML induces gut microbiota dysbiosis

As previous studies revealed the importance of gut microbiota and the close relationship between the metabolic syndrome and gut microbiota dysbiosis [21, 22], we further examined the alteration of the diversity and composition on gut microbiota between the GML and CON groups. No significant differences were observed for α-diversity (Chao 1’s diversity parameter, observed species and Shannon’s diversity parameter, respectively) between the GML and CON groups (Fig. 3a). Furthermore, the Venn diagram demonstrated that 640 of OTUs were common in the GML and CON groups, and the number of their unique OTUs was 58 and 66 respectively (Fig. 3a). However, a significant decrease for β-diversity in the GML group was observed, compared to the CON group (Fig. 3b). In order to evaluate the structure alteration of gut microbiota between the GML and CON groups based on differences in phylogenetic diversity, three-dimensional principal coordinate analysis (PCoA) plot were constructed for the evaluation of the dissimilarity and the community composition between samples from weighted UniFrac distances. Interestingly, PCoA plot showed that the samples between the GML and CON groups was not rather different but significant \( P = 0.033 \) at the Week 8, meanwhile the samples in the GML group were clustering intensively and gradually away from the CON group, suggesting that GML altered the communities of gut microbiota in a characteristic direction (Fig. 3b).

The gut bacterial communities among the GML and CON groups at the Week 8 were dominated by two phyla: Bacteroidetes and Firmicutes, and no significant differences were observed (Fig. 3c). However, the relative abundance of LPS-suppressing phyla Verrucomicrobia in the GML group showed a significant decrease, compared to the CON group \( P = 0.009 \). Furthermore, the CON group consisted of an obviously higher relative abundance of sequences belonging to the LPS-producing phyla Proteobacteria (5.86%) than the GML group.
(4.46%) \((P = 0.075)\), but no significant difference was observed (Fig. 3c). The core gut microbiota identified in two groups was similar and comprised of 33 genera (the relative abundance of OTUs > 0.01%) (Fig. 4a).

Bacteroidetes were dominated by the genera *Bacteroides, Parabacteroides, Prevotella, [Prevotella], AF12, Odoribacter, Mucispirillum, Paraprevotella and Rikenella*. The Firmicutes were dominated by the genera *Oscillospira, Clostridium, Allobaculum, Dehalobacterium, Coprococcus, Anaerostipes, Ruminococcus, [Ruminococcus], Lactobacillus, Turicibacter, Butyricicoccus, Roseburia and Dorea*. The phyla *Verrucomicrobia* was dominated by the genus *Akkermansia*. The phyla *Proteobacteria* was dominated by the genera *Helicobacter, Desulfovibrio, Bradyrhizobium, Sutterella, Bilophila, Mesorhizobium, Bilophila, Escherichia and Lupinus*. In addition, the genera *Bifidobacterium* and *Adlercreutzia* belonged to the phyla *Actinobacteria*.

Interestingly, the anti-inflammatory genus *Akkermansia* and *Lupinus* in the GML group was significantly decreased \((P = 0.009)\), while the pathogenic genus *Turicibacter, Bradyrhizobium* and *Escherichia* in the GML group were significantly increased \((P = 0.016, P = 0.004, and P = 0.047, respectively)\) compared to those of the CON group (Fig. 4a). However, the butyric acid producing bacteria genera *Roseburia* was significantly increased \((P = 0.004)\) compared to that of the CON group. In order to further understand the effects of GML on the induction of gut microbiota dysbiosis, we identified the significantly enriched or depleted species in the GML group compared with the CON group (Fig. 4b). Importantly, *Akkermansia muciniphila* and *Lupinus luteus* in the GML group were significantly decreased by 29.9 times \((P = 0.009)\) and 3.6 times \((P = 0.013)\), respectively, whereas *Bacteroides acidifaciens* and *Escherichia coli* in the GML group were significantly increased by 1.5 times \((P = 0.036)\) and 4.3 times \((P = 0.046)\), respectively, compared to those in the CON group.
The predictive functions of the microbiota genes were shown in Fig. 5. The different metabolism pathways belonged to the carbohydrate metabolism, amino acid metabolism and lipid metabolism. Obviously, GML significantly increased the DNA abundances of 10 predicted metabolism pathways of butanoate metabolism \( (P = 0.037) \), propanoate metabolism \( (P = 0.028) \), valine, leucine and isoleucine degradation \( (P = 0.007) \), tryptophan metabolism \( (P = 0.015) \), lysine degradation \( (P = 0.014) \), beta-alanine metabolism \( (P = 0.018) \), cyanoamino acid metabolism \( (P = 0.030) \), fatty acid metabolism \( (P = 0.013) \), glycerolipid metabolism \( (P = 0.041) \), synthesis and degradation of ketone bodies \( (P = 0.004) \), compared to those of the CON group.

### 3.3 GML increases serum LPS concentration and systemic low-grade inflammation

Finally, we determined whether dietary GML affected serum LPS concentration and systemic low-grade inflammation, which are commonly derived from gut microbiota dysbiosis. Notably, a significant increase \( (P < 0.001) \) was observed for the serum LPS concentration (61.1 times) in the GML group at the Week 8 compared to the CON group (Fig. 6a). Accordingly, the circulating levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in the GML group were significantly increased \( (P = 0.034, P = 0.002 \text{ and } P < 0.001, \text{ respectively}) \) (Fig. 6b-d). In contrast, the production of anti-inflammatory cytokine IL-10 in the GML group was largely reduced \( (P = 0.175) \) (Fig. 6e).

### 4 Discussion
The consumption of GML used as the food antimicrobial-emulsifier in food industry is steadily increasing, as it is on the FDA’s Generally Recognized as Safe (GRAS) list, which suggests no acute toxicity and/or no promotion of cancer, but supporting scientific data remain sparse for chronic diseases. In the present study, we observed that GML induced mouse metabolic syndrome, gut microbiota dysbiosis and systemic low-grade inflammation in low fat diet (Fig. 7), which indicated the potential of GML for inducing chronic diseases. Recently, growing evidence has indicated the relationship between broad use of emulsifying agents and increased societal incidence of metabolic syndrome. The NCEP–ATP III proposed that the features of metabolic syndrome were abdominal obesity, raised serum triglycerides and fasting plasma glucose, lower high-density lipoprotein (HDL)-cholesterol and increased blood pressure, and abdominal obesity played a central role in metabolic syndrome. In this study, GML administration resulted in significant increases in the body weight, food intake, weight gain, body fat percentage, epididymal fat, serum TG and LDL concentrations, significant enlargement in the size of stained fat droplets in epididymal adipose tissue, and significant decreases in the body muscle percentage, liver weight and serum HDL concentrations (Fig. 1and 2) compared to the CON group, indicating that the dietary GML induced metabolic syndrome in mice model.

Emulsifier-induced metabolic syndrome was associated with gut microbiota dysbiosis. As already demonstrated in previous studies [11, 23], dietary supplementation with non-caloric artificial sweetener (saccharin) and emulsifiers (CMC and P80), significantly promoted the metabolic syndrome by altering the diversity, composition and function of gut microbiota in mice. Our study revealed that GML significantly reduced the β-diversity parameter of gut microbiota at the Week 8 compared to the CON group (Fig. 3a and b).
Interestingly, PCoA plot demonstrated the samples between the GML and CON groups is not rather different but significant ($P = 0.033$) at the Week 8, meanwhile the samples in the GML group were clustering intensively and gradually away from the CON group, suggesting that GML altered the communities of gut microbiota in a characteristic direction (Fig. 3b). It is worth noting that the LPS-suppressing phyla Verrucomicrobia in the GML group was significantly increased, and the LPS-producing phyla Proteobacteria was obviously lower compared to those in the CON group (Fig. 3c). Likewise, our results that the LPS-suppressing genus Akkermansia and Lupinus in the GML group was significantly decreased, while the LPS-producing genus Turicibacter, Bradyrhizobium and Escherichia in the GML group was significantly increased (Fig. 4a), indicated that GML induced primary gut microbiota dysbiosis, at least in part, through significantly reducing LPS-suppressing bacteria (those which can lower the numbers of LPS-producing bacteria), and increasing LPS-producing bacteria. These findings are consistent with the results of Kanakaraju et al [16], who demonstrated that increasing LPS-producing bacteria (e.g. E. coli) and decreasing LPS-suppressing bacteria (e.g. Bifidobacterium) in gut resulted in metabolic syndrome. Much progress has been made in the identification of LPS-suppressing genera and LPS-producing genera, revealing that LPS-producing families/genera are presented in several families/genera (Enterobacteriaceae, Clostridium cluster XI, Prevotella and SFB), and Lactobacillus, Bifidobacterium, Enterococcus faecium, Clostridium cluster XIVa, Clostridium cluster IV and Akkermansia are mainly LPS-suppressing genera [24, 25], which have been linked to reducing the intestinal permeability and inflammatory [26], exerting the anti-obesity effects [14] and stimulating the host’s immune system and metabolism [27], and their abundance was correlated significantly with serum LPS levels [28].
Systemic low-grade inflammation is characterized by elevated circulating levels of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 [29-31] and primarily caused by a gradual increase in plasma endotoxins, particularly LPS. The binding with Toll-like receptor-4 (TLR4) and subsequent activation of NLRP3 inflammasome leads to the increased expression of inflammatory cytokines [32, 33]. As a consequence, our results that significant increases in the serum LPS concentration, pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the GML group (Fig. 6), suggested that GML significantly induced systemic low-grade inflammation. Importantly, the serum LPS concentration in GML group was significantly increased, compared to the CON treatment, in accordance with the increase of the LPS-producing bacteria genera and decrease of the LPS-suppressing bacteria genera, indicating that the serum LPS is an important link in the cross-talk between the gut microbiota and the host inflammation, and the supplementation of GML might induce gut LPS-relative microbiota dysbiosis to increase in the serum LPS concentration and further result in systemic low-grade inflammation. Recently, several studies have highlighted that systemic low-grade inflammation resulted from gut dysbiosis, which is now considered as a critical pathological factor underlying many modern chronic diseases, including diabetes, cardiovascular disease, cancer, neurodegenerative diseases and aging [29, 30]. Thus, a better understanding of the effects of dietary GML for modulating gut microbiota would be important for evaluating the safety of its use in consumed foods.

In order to further understand the effects of GML on the regulation of gut microbiota, we identified the significantly enriched or depleted species and different functional genes of bacteria in the GML group compared with the CON group (Fig. 4b and Fig. 5). Specific and significant increase of Akkermansia muciniphila (widely considered as mucos-protection bacterium) [34-36] in the gut was important for reducing the host...
susceptibility to metabolic syndrome and systemic low-grade inflammation, but our results suggested that
GML could significantly decrease Akkermansia muciniphila and Lupinus luteus, and increase Bacteroides 
acidifaciens and Escherichia coli, which were adverse to reduce the serum LPS concentration and
pro-inflammatory cytokines [26], and might result in obesity and metabolic endotoxemia [27]. Bacteroides 
acidifaciens has recently been shown to have an important role in the prevention against obesity, the
improvement of insulin sensitivity in mice and the production of immunoglobulin (IgA) in the large intestine of
mice [37, 38]. This production plays an adaptive role in the intestinal mucosal immune system [39]. Since IgA is
enhanced in metabolic disorders [40], the relative abundance of Bacteroides acidifaciens in the GML group
may be associated with the role of gut microbiota in the inflammatory signalling peculiar to metabolic
syndrome [41]. PICRUSt function prediction further indicated that the gut microbiota in the GML group had a
significantly higher capacity of the carbohydrate metabolism, amino acid metabolism and lipid metabolism,
which would increase host energy harvest contributing to an increased incidence of obesity/metabolic
syndrome and other chronic inflammatory diseases [11]. In summary, these findings indicated that the
relatively low-dose GML in low fat diet induced metabolic syndrome, gut microbiota dysbiosis and systemic
low-grade inflammation.

In conclusion, the present study indicates that dietary GML in low fat diet had the potential to induce
metabolic syndrome, gut microbiota dysbiosis and systemic low-grade inflammation. Our study supports the
emerging concept that emulsifier-induced gut microbiota dysbiosis are contributing to an increased incidence
of obesity/metabolic syndrome and other chronic inflammatory diseases. However, the underlying mechanism
of the induction of metabolic syndrome, gut microbiota dysbiosis and systemic low-grade inflammation by GML needs to be further explored.

Z.L.J., H.Z. and F.Q.F. conceptualized the research; Z.L.J., M.J.Z., H.Z., Y.L. and M.Y.L participated in performed experiments and analyzed data; Z.L.J., H.Z. and F.Q.F. wrote the original draft; Z.L.J., M.J.Z., H.Z., Y.L. and M.Y.L wrote, reviewed, and edited the work; F.Q.F. were involved in funding acquisition; F.Q.F. provided the resources for the work; Z.L.J., H.Z. and F.Q.F. supervised the work.

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The authors have declared no conflict of interest.

5 References


different mechanism than in the small intestine: Bacteroides acidifaciens promotes IgA production in the large intestine by inducing germinal center formation and increasing the number of IgA+ B cells. *Immunobiology* 2013, 218, 645-651.


Figure captions

Figure 1. GML increased body weight, food intake and fat accumulation in low-fat diet fed mice. Effects of GML treatment (n = 10/group) on (a) body weight, food intake (n = 4, 4 cages/group), weight gain, body fat percentage, body muscle percentage, epididymal fat, (b) the size of stained fat droplets in epididymal adipose tissue (n = 5/group), and (c) Liver lipid content assessed using H&E staining and liver weight (n = 5/group).

Scale bar, 30 μm. Data are expressed as mean ± SE. Value with asterisk is significantly different according to one-way ANOVA with Tukey test or unpaired two-tailed T-test (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 2. GML exacerbated serum TG, LDL and HDL disorder. (a) Serum triglycerides (TG), total serum cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), fasting plasma glucose, fasting insulin (n = 10/group), and (b) Atherogenic index and homeostasis model assessment-insulin resistance index (HOMA-IR) (n = 10/group). Data are expressed as mean ± SE. Value with asterisk is significantly different according to the unpaired two-tailed T-test (*P < 0.05, **P < 0.01, ***P < 0.001).
**Figure 3.** GML altered the diversity and communities of gut microbiota. (a) α-diversity: Observed species, Chao 1’s diversity parameter, Shannon’s diversity parameter and Venn Diagram between treatments (n = 10/group), (b) three-dimensional principal coordinate analysis (PCoA) plot based on weighted UniFrac distances and β-diversity of gut microbiota (n = 10/group), and (c) relative abundance of gut microbiota at the phylum level (n = 10/group). Data are expressed as mean ± SE. Value with asterisk is significantly different according to the unpaired two-tailed T-test (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 4. GML altered the composition of gut microbiota. (a) Relative abundance of gut microbiota at the genera level (those with > 0.1% are represented) (n = 10/group), and (b) Significantly enriched or depleted species in the GML group at the week 8 compared to those in the CON group (n = 10/group). Data are expressed as mean ± SE. Value with asterisk is significantly different according to the unpaired two-tailed T-test (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 5. GML altered the predicted metabolism pathways of gut microbiota. The DNA abundances of KEGG pathways in level-3 functional prediction by PICRUSt (n = 10/group). Data are expressed as mean ± SE. Value with asterisk is significantly different according to the unpaired two-tailed T-test (*P < 0.05, ** P < 0.01, ***P < 0.001).
Figure 6. GML up-regulated the production of the serum LPS concentration and pro-inflammatory cytokines.

Serum LPS concentration (a), IL-1β (b), IL-6 (c), TNF-α (d) and IL-10 (e) (n = 6/group). Data are expressed as mean ± SE. Value with asterisk is significantly different according to the unpaired two-tailed T-test (*P < 0.05, ** P < 0.01, ***P < 0.001).
Figure 7. Diagram illustrating a proposed mechanism by which GML induced metabolic syndrome, gut microbiota dysbiosis and systemic low-grade inflammation in low-fat diet fed mice. A relatively low-dose GML up-regulates LPS-producing bacteria (e.g. E. coli) while decreases LPS-suppressing bacteria (e.g. A. muciniphila) resulting in gut microbiota dysbiosis and metabolic dysfunction. These changes increase LPS production resulting in increased metabolic endotoxemia. The subsequent increases of blood inflammatory cytokines leads to the promotion of systemic low-grade inflammation and metabolic syndrome. The dotted arrows represent a mechanism that requires further proof.
Graphic Abstract

GML induced metabolic syndrome, gut microbiota dysbiosis and circulating low-grade inflammation by significantly increasing the body fat percentage, epididymal fat, exacerbating serum lipid profiles disorder and histopathologic abnormality of epididymal adipose tissue, changing the composition and metabolic function of gut microbiota and up-regulating the production of the serum LPS concentration and circulating inflammatory cytokines mainly involved in IL-1β, IL-6 and TNF-α. The dotted arrows represent a mechanism that requires further proof.
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