Dual probiotic strains suppress high fructose-induced metabolic syndrome

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AIM: To investigate the effect of novel probiotics on the clinical characteristics of high-fructose induced metabolic syndrome.

METHODS: Male Wistar rats aged 4 wk were fed a 70% w/w high-fructose diet (n = 27) or chow diet (n = 9) for 3 wk to induce metabolic syndrome, the rats were then randomized into groups and administered probiotic \( \text{Lactobacillus curvatus} \) (\( L. \) curvatus) HY7601 and \( \text{Lactobacillus plantarum} \) (\( L. \) plantarum) KY1032 at \( 10^9 \) cfu/d or \( 10^{10} \) cfu/d or placebo by oral gavage for 3 wk. Food intake and body weight were measured once a week. After 6 wk, the rats were fasted for 12 h, then anesthetized with diethyl ether and sacrificed. Blood samples were taken from the inferior vena cava for plasma analysis of glucose, insulin, C-peptide, total-cholesterol, triglycerides and thiobarbituric acid-reacting substances. Real-time polymerase chain reaction was performed using mouse-specific Taqman probe sets to assess genes related to fatty acid \( \beta \)-oxidation, lipogenesis and cholesterol metabolism in the liver. Target gene expression was normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase.

RESULTS: Rodents fed a high-fructose diet developed clinical characteristics of the metabolic syndrome including increased plasma glucose, insulin, triglycerides, total cholesterol and oxidative stress levels, as well as increased liver mass and liver lipids compared to chow fed controls. Probiotic treatment (\( L. \) curvatus HY7601 and \( L. \) plantarum KY1032) at high (\( 10^{10} \) cfu/d) or low dosage (\( 10^9 \) cfu/d) lowered plasma glucose, insulin, triglycerides and oxidative stress levels. Only high-dose probiotic treatment reduced liver mass and liver cholesterol. Probiotic treatment reduced lipogenesis via down-regulation of SREBP1, FAS and SCD1 mRNA levels and increased \( \beta \)-oxidation via up-regulation of PPAR\( \alpha \) and CPT2 mRNA levels.

CONCLUSION: Probiotic \( L. \) curvatus HY7601 and \( L. \) plantarum KY1032 combined suppressed the clinical characteristics of high-fructose-induced metabolic syndrome, therefore, may provide a natural alternative for the treatment of diet-induced metabolic syndrome.

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Key words: Dyslipidemia; Fasting glucose; Gut microbiota; High-fructose diet; Inflammation; Insulin resistance; Lactobacillus; Metabolic syndrome; Oxidative stress; Probiotic

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INTRODUCTION

Metabolic syndrome is a rapidly growing worldwide pandemic, which is associated with a greater risk of multiple chronic pathologies including cardiovascular disease and Type 2 diabetes. Metabolic syndrome is characterised by a cluster of metabolic abnormalities including insulin resistance, elevated fasting glucose, elevated plasma triglycerides, elevated blood pressure, low-grade inflammation, abdominal obesity and reduced high-density lipoprotein (HDL)-cholesterol[1-2]. There is no universal cause of metabolic syndrome, although major underlying risk factors include abdominal obesity, physical inactivity or diet-induced diabetes[3].

Diabetogenic diets with high fructose content have been strongly implicated in the development of metabolic syndrome, cardiovascular disease and Type 2 diabetes[3]. Food manufacturers are increasingly using fructose corn syrup to increase sweetness, as well as the palatability of food and beverages. However, there is growing experimental evidence that excessive fructose intake can lead to metabolic abnormalities, including insulin resistance, dyslipidemia as well as abdominal adiposity in both animals and humans[3,6]. Animals fed a high-fructose diet develop clinical characteristics of metabolic syndrome, therefore, high-fructose fed animals are particularly useful for assessing potential therapeutic interventions against metabolic syndrome[3,5]. Excess dietary fructose can be converted to triglycerides through de novo lipogenesis, resulting in increased lipid accumulation in the liver and elevated blood lipid levels. Over a period of time prolonged high intracellular and systemic lipid levels can cause increased oxidative stress and inflammation, both of which can trigger insulin resistance, leading to increased blood glucose levels. Due to the lack of effective drugs to treat metabolic syndrome, there is growing interest in natural therapeutics to prevent or manage metabolic syndrome.

Over the past five years, probiotics have rapidly emerged as natural therapeutics with potential to target key risk factors associated with metabolic syndrome[6,7]. Probiotics consist of single or multiple live bacterial species, which may directly or indirectly modulate gut microbial activity and improve host health. The human gut harbours between 10^{14} bacterial species collectively forming the gut microbiota[9]. Gut microbial communities are proposed to provide the host with the ability to harvest otherwise inaccessible energy from the diet[9,10] and modulate host genes associated with energy storage in adipose tissue[3,12]. Probiotics have been widely assessed in-vivo in diet-induced obesity models[13-17], however, different probiotic species even from the same family can exert variable effects on lipid accumulation and obesity[18], therefore, it remains essential to assess the effectiveness of probiotic strains in different animal disease models in-vivo. Probiotic yogurt containing multiple Lactobacillus strains has been reported to alleviate fasting blood glucose, plasma insulin and triglyceride in high-fructose fed rats[19]. However, few other studies have considered the impact of probiotics on high-fructose diet-induced metabolic syndrome. To our knowledge, the dose-dependent and metabolic effects of probiotic treatment in high-fructose-induced metabolic syndrome remain to be established.

The aim of this study was to assess the dose-dependent effects of a probiotic consisting of Lactobacillus curvatus (L. curvatus) HY7601 and Lactobacillus plantarum (L. plantarum) KY1032 on hyperlipidemia, hyperglycemia, insulin resistance, oxidative stress and hepatic metabolism related gene expression in high-fructose fed rats with metabolic syndrome. We hypothesized that probiotic treatment may protect against dysregulated metabolism induced by a high-fructose diet in a dose-dependent manner.

MATERIALS AND METHODS

Animals, diets and experimental design

Male Wistar rats (n = 36) aged 4 wk were purchased from Jackson Laboratories (Bar Harbor, United States). All rats were individually housed under a constant temperature and humidity (22 ± 1 °C, 55% ± 10%) with a 12 h light/12 h dark cycle. The experimental design consisted of a pretreatment phase (0-3 wk) and a treatment phase (3-6 wk). During the pretreatment phase, male Wistar rats were fed a 70% w/w high-fructose diet (n = 27) to induce metabolic abnormalities or a chow diet (n = 9) for 3 wk. The composition of the high-fructose diet was formulated according to Table 1. During the treatment phase, the placebo (HF) group (n = 9), low dose probiotic (LP) group (n = 9) and high dose probiotic (HP) group (n = 9) were fed the same high-fructose diet with placebo, 10^7 cfu probiotics or 10^8 cfu probiotics, respectively, administered orally each day for a further 3 wk. The chow control group was fed the same chow diet with placebo administered orally each day for a further 3 wk. Freeze-dried Lactobacillus strains were produced by Culture Systems Inc. (United States), and packed with lactose according to Table 2. Each pack was resuspended in 500 μL distilled water prior to administration. Food intake and body weight were measured once a week. Before sacrifice, rats were fasted for 12 h and anesthetized with diethyl ether. Blood samples were taken from the inferior vena cava for plasma analysis. Epididymal adipose tissue and liver tissue were removed, rinsed with phosphate buffered saline, weighed and immediately frozen at -70 °C. The experimental design was approved by the Ethics Committee of Korea Yakult Company Limited R and D Center.

Blood analysis

Plasma glucose, insulin and C-peptide concentrations
were determined using the glucose assay kit (Cayman, United States), insulin enzyme-linked immunosorbent assay (ELISA) kit (Millipore, United States) and rat C-peptide ELISA kit (EIAab, China), respectively, according to the manufacturer’s instructions. Insulin resistance was assessed based on homeostasis model assessment of insulin resistance (HOMA-IR), calculated as the product of fasting plasma glucose (FPG) and insulin (FPI), divided by a constant. The equation was \[ \text{HOMA-IR} = \frac{\text{FPG} \times \text{FPI}}{2430}. \] Plasma total-cholesterol and triglyceride concentrations were determined using commercial kits (AsanPharm, South Korea). Plasma thiobarbituric acid-reacting substances (TBARS) were measured to assess oxidative stress as described previously.

**Hepatic lipid profile analysis**

Hepatic lipids were extracted as previously reported. The dried lipid residues were dissolved in 1 mL of isopropanol for the triglyceride and cholesterol assays. Hepatic triglyceride and cholesterol concentrations were measured using the same commercial kits (AsanPharm, South Korea). Plasma thiobarbituric acid-reacting substances (TBARS) were measured to assess oxidative stress as described previously.

**RQPCR**

Total RNA was extracted from liver (15 mg) tissue using an RNAaqueous kit (Ambion, United States). DNA was removed with a Turbo DNA-free kit (Ambion, United States). RNA integrity was verified and RNA quantified using a GeneQuant Pro spectrophotometer (GE Healthcare, United States). Total RNA (2 μg) was reverse-transcribed into cDNA with a high-capacity RNA-to-cDNA kit (Applied Biosystems Inc., United States). Then cDNA was amplified on a 7500 Real Time PCR System (Applied Biosystems Inc., United States) using mouse-specific Taqman probe sets (Table 3) under the following conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Target gene expression was normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (catalog number Rn01775763_g1). Data was analyzed using the ABI 7500 System Sequence Detection software (Applied Biosystems Inc., United States) and presented as mean ± SE.

**Statistical analysis**

All data were presented as mean ± SE. Statistical analysis was performed using SPSS software (SPSS Inc., United States). Data were analyzed by one way analysis of variance, and the differences between experimental groups were evaluated using Duncan’s multiple range test at the \( P < 0.05 \) level. Significant differences between chow control and HF control groups were determined using unpaired Student’s \( t \)-test. Significant differences between week 3 and week 6 in each parameter were determined using the paired Student’s \( t \)-test, and the values were considered statistically significant when \( P < 0.05 \).

**RESULTS**

**Effects of probiotic treatment on food intake, body weight and tissue mass**

Metabolic syndrome was induced in rodents by feeding them a high-fructose diet over 3 wk, while a control group was fed a chow diet. Rodents with metabolic syndrome were then randomized into three treatment groups and fed a high-fructose diet for a further 3 wk, with daily treatment of either probiotic (HP) at a dose of \( 1 \times 10^{9} \) cfu/d, or placebo administered by oral gavage. The chow control group were fed the same diet for the same period and administered placebo by oral gavage. Average food intake was suppressed in the high-fructose diet fed rodents compared to the chow fed controls (Figure 1A). However, low or high dose probiotic treatment had no significant effect on food intake (Figure 1A). Body weight gain and epididymal fat mass were not significantly affected by high-fructose feeding or probiotic treatment regardless of dosage (Figure 1B and C). Importantly, average liver
fed rats were significantly higher than that of the chow fed controls at week 6, but were effectively reduced following LP and HP treatment by 24% ($P < 0.05$) and 14% ($P < 0.05$), respectively, (Figure 3A). The average plasma insulin levels of the high-fructose fed rats were significantly higher at both weeks 3 and 6 compared to the chow fed controls. However, after HP treatment plasma, insulin levels were substantially lower (31%, $P < 0.05$) compared to the high-fructose fed rats. Moreover, the plasma insulin levels following LP and HP treatment were reduced by 33% ($P < 0.01$) and 29% ($P < 0.05$), respectively, compared to before treatment (Figure 3B).

The HOMA-IR, a representative index of insulin resistance, was significantly higher in the high-fructose fed rats compared to the chow fed controls at week 6, but was significantly reduced by LP (35%) and HP (34%) treatment (Figure 3C). Furthermore, HOMA-IR following LP treatment was 25% ($P < 0.05$) lower compared to before treatment (Figure 3C). Plasma C-peptide is another biomarker associated with insulin resistance.

mass, which was significantly increased by high-fructose diet, was significantly lower following HP treatment by 10% ($P < 0.01$) compared to high-fructose fed controls (Figure 1D).

**Effects of probiotic treatment on dyslipidemia**

Hypertriglyceridemia was effectively induced by a high-fructose diet within 3 wk (163.91 ± 6.50 mg/dL vs 49.26 ± 3.99 mg/dL, HF and chow group, respectively). However, subsequently 3-wk of probiotic treatment significantly reduced average plasma triglyceride levels by 46% compared to placebo treatment in the high-fructose fed rodents (Figure 2A). The probiotic treatment had no influence on either total cholesterol or HDL cholesterol levels, which were increased by high-fructose diet intake (Figure 2B and C).

**Effects of probiotic treatment on hyperglycemia, insulin resistance and oxidative stress**

The average plasma glucose levels of the high-fructose fed rats were significantly higher than that of the chow fed controls at week 6, but were effectively reduced following LP and HP treatment by 24% ($P < 0.05$) and 14% ($P < 0.05$), respectively, (Figure 3A). The average plasma insulin levels of the high-fructose fed rats were significantly higher at both weeks 3 and 6 compared to the chow fed controls. However, after HP treatment plasma, insulin levels were substantially lower (31%, $P < 0.05$) compared to the high-fructose fed rats. Moreover, the plasma insulin levels following LP and HP treatment were reduced by 33% ($P < 0.01$) and 29% ($P < 0.05$), respectively, compared to before treatment (Figure 3B). The HOMA-IR, a representative index of insulin resistance, was significantly higher in the high-fructose fed rats compared to the chow fed controls at week 6, but was significantly reduced by LP (35%) and HP (34%) treatment (Figure 3C). Furthermore, HOMA-IR following LP treatment was 25% ($P < 0.05$) lower compared to before treatment (Figure 3C). Plasma C-peptide is another biomarker associated with insulin resistance.
Plasma C-peptide level was 24% ($P < 0.05$) lower following HP treatment compared to placebo treatment in high-fructose fed rats (Figure 3D). Moreover, plasma C-peptide levels after HP treatment were 28% ($P < 0.05$) lower compared to before treatment (Figure 3D). In addition, probiotic treatment of either LP or HP decreased plasma TBARS levels by 37% ($P < 0.01$) and 50% ($P < 0.001$), respectively (Figure 3E).

**Effects of probiotic treatment on hepatic lipid content and gene expression**

Both hepatic triglyceride and cholesterol levels in the high-fructose fed rats were significantly higher than those in the chow fed controls. Probiotic treatment with either LP or HP tended to reduce hepatic triglyceride levels (Figure 4A), and HP significantly reduced (27%, $P < 0.05$) hepatic cholesterol levels compared to the high-fructose fed controls (Figure 4B).

In order to determine the mechanisms underlying the probiotic effect on hepatic lipid homeostasis, we examined the mRNA levels of hepatic genes associated with lipid metabolism (Figure 5A-C). High-fructose diet intake altered gene expression compared to chow diet intake. The changes in gene expression in the high-fructose group indicated decreases in fatty acid β-oxidation (PPARα, CPT1, CPT2 and ACOX1), cholesterol uptake (LDLR) and bile acid synthesis (CYP7A1), and increases in fatty acid synthesis (SREBP1, FAS, SCD1). Importantly, HP treatment significantly reversed high-fructose-induced gene expression changes including up-regulation of PPARα (+76%), CPT2 (+66%) and CYP7A1 (+71%), and down-regulation of SREBP1 (-30%), FAS (-54%) and SCD1 (-23%), although LP treatment did not cause any significant changes in hepatic lipid metabolism gene expression.

**DISCUSSION**

Recent studies indicate that the gut microbiota plays an important role in host lipid and glucose metabolism. Therefore, therapeutic probiotics which can manipulate the gut microbiota may also prevent some of the risk factors underlying the development of metabolic syndrome including dyslipidemia, elevated fasting glucose levels and insulin resistance[23]. In the present study, we used a novel probiotic consisting of *L. curvatus* HY7601 and *L. plantarum* KY1302 isolated from Korean fermented cabbage. We showed that probiotic administered to high-fructose fed mice reversed the risk factors underlying the metabolic syndrome. Previous evidence from high-fructose diet-fed rat studies indicated that a probiotic-cultured yogurt called Dahi can also improve metabolic abnormalities[19,24]. Importantly, in the present study, we established the dose-dependent effects of a novel combination of probiotic strains on metabolic syndrome.

High-fructose intake is reported to promote lipogenesis and suppress glucose intake[25-27]. Consistent with other studies of high-fructose fed animals, hepatic gene expression analysis indicated that lipogenesis was increased via...
upregulation of SREBP1, FAS and SCD1, conversely β-oxidation was decreased via downregulation of PPARα and PPARγ-regulated CPT1, CPT2 and ACOX1 expression. Increased lipogenesis and decreased β-oxidation lead to excess accumulation of cellular lipids, as shown by the liver enlargement and hypertriglyceridemia in the high-fructose fed rats in the present study. Hypertriglyceridemia is known to be an important predictor of cardiovascular disease.

Figure 3  Effects of probiotic treatment on plasma glucose, plasma insulin, insulin resistance, plasma C-peptide and plasma thiobarbituric acid-reacting substances in high-fructose diet-fed rats. Results are expressed as mean ± SE. *P < 0.05 and **P < 0.01 HF control vs chow control by unpaired Student’s t-test; †P < 0.05 and ‡P < 0.01 week 6 vs week 3 by paired Student’s t-test; Bars with different capital letters are significantly different at P < 0.05 by Duncan’s multiple range tests. HF: High fructose diet; LP: Low dose probiotic; HP: High dose probiotic; TBARS: Thiobarbituric acid-reacting substances.
diabetes, cardiovascular disease mortality in subjects with diabetes or impaired glucose tolerance, therefore, reducing plasma triglyceride levels may improve long-term health.

Previous studies have shown that probiotics can alter the gut microbiota, and direct gut microbiota manipulations in germ-free mice significantly affect host lipid metabolism. Here we showed that 10^10 cfu/d probiotic treatment led to upregulated PPARα and CPT2 expression reflecting activation of β-oxidation, and down-regulated SREBP1, FAS and SCD1 expression reflecting suppression of lipogenesis. Moreover, these probiotics induced transcriptional changes which resulted in a significant reduction in liver mass and plasma triglyceride levels. These findings are consistent with a previous report which showed that L. plantarum KY1032 inhibits lipid droplet accumulation during adipocyte differentiation. In contrast to plasma triglyceride levels, hepatic triglyceride levels were only slightly reduced by probiotic treatment. We hypothesized that the probiotic-induced increase in hepatic β-oxidation related gene expression was partly to clear excess hepatic triglycerides generated through high-fructose-induced de novo lipogenesis.

Contrary to hepatic triglyceride levels, cholesterol levels were significantly reduced by probiotic treatment, whereas plasma cholesterol levels were unchanged. We assessed whether the reduction in hepatic cholesterol following probiotic treatment was due to altered hepatic cholesterol metabolism related gene expression. High-fructose diet suppressed CYP7A1 expression, which encodes cholesterol 7 α-hydroxylase, the rate-limiting enzyme involved in the formation of bile from cholesterol. However, 10^10 cfu/d probiotic treatment upregulated CYP7A1 expression indicating increased bile synthesis activity. Some probiotics are also reported to increase bile salt hydrolase activity. Hepatic LDLR expression remained unchanged, suggesting that probiotic treatment did not enhance uptake of plasma cholesterol into the liver, which is consistent with the absence of changes in plasma cholesterol levels. However, the effect of probiotics on liver cholesterol metabolism may be partly dependent on the diet regime used, because in high-fat or high-cholesterol diet fed animals, probiotics are reported to decrease plasma and hepatic cholesterol levels together.

The probiotic effect on host lipid levels may also contribute to the observed improvement in glucose homeostasis. Excess intracellular lipids can inhibit insulin signalling, hence reduce insulin-stimulated glucose uptake leading to hyperglycemia and hyperinsulinemia as shown in this study. We observed that probiotic treatment at 10^10 or 10^10 cfu/d significantly lowered plasma glucose, insulin and C-peptide concentrations, and reduced insulin resistance indicated by the reduced HOMA-IR index. Excess fructose intake is also reported to promote lipid peroxidation and oxidative stress is implicated in the pathogenesis of insulin resistance. In the present study, probiotic treatment at 10^10 or 10^10 cfu/d significantly reversed the oxidative stress present in the high-fructose fed rats with metabolic syndrome, indicated by lower plasma TBARS levels. Some Lactobacillus strains are also reported to possess anti-oxidative activity. For example, probiotics have been reported to reduce exercise-induced oxidative stress, via increases in anti-oxidative activity, which helps neutralize reactive oxygen species. Excess fructose intake is also reported to promote lipid peroxidation and oxidative stress is implicated in the pathogenesis of insulin resistance. In the present study, probiotic treatment at 10^10 or 10^10 cfu/d significantly lowered plasma glucose, insulin and C-peptide concentrations, and reduced insulin resistance indicated by the reduced HOMA-IR index. Excess fructose intake is also reported to promote lipid peroxidation and oxidative stress is implicated in the pathogenesis of insulin resistance.
free mice transplanted with a small artificial gut microbial community, a multi-species probiotic failed to change the gut microbiota\[33\]. Whether multi-species probiotics are more effective than single-species probiotics is not clear, because few studies make this comparison. We used a two species probiotic in the present study, and based on our preliminary data the results suggest that combined species are more effective than either species alone. Finally, whether probiotics exert dose-dependent effects against metabolic syndrome has not been evaluated, nor has the minimum amount of live bacteria that is necessary for functional health benefits. Evidence from the present study indicates that probiotics containing 10^10 cfu/d showed the greatest effectiveness against high-fructose-induced metabolic syndrome over a relatively short period, without any adverse effects. In contrast, probiotic treatment at 10^7 cfu/d exerted minimal effects on hypertriglyceridemia, but effectively improved hyperglycaemia and insulin resistance. In the case of hepatic gene expression, 10^9 cfu/d probiotic treatment had no modulatory effect on any of the genes tested, but probiotic treatment at 10^10 cfu/d modulated lipogenesis and β-oxidation related genes.

In conclusion, probiotic *L. curvatus* HY7601 and *L. plantarum* KY1032 combined can suppress the clinical characteristics of high-fructose-induced metabolic syndrome, therefore, may provide a natural alternative for the treatment of diet-induced metabolic syndrome.

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Choi MS and McGregor RA declare no conflicts of interest. Park DY, Ahn YT and Huh CS are current or past employees of Korea Yakult Co., Ltd.

**COMMENTS**

**Background**

Metabolic syndrome is a growing health problem characterised by elevated blood sugar and blood lipid levels, insulin resistance, high blood pressure and abdominal obesity. Poor diet consisting of high sugar or high fat content is a major risk factor for metabolic syndrome. There is no universal treatment for metabolic syndrome. Probiotics found in fermented foods have emerged as a natural way of protecting against metabolic syndrome, but many probiotic strains have varying metabolic effects. Therefore, a major challenge for scientists is discovering probiotic strains, which may help protect against metabolic syndrome.

**Research frontiers**

Probiotic strains identified in kimchi, a traditional Korean fermented cabbage consumed regularly in South Asian countries, are reported to have various beneficial properties. Current research aims to determine whether these probiotic strains are effective in different animal models of disease including metabolic syndrome. The authors showed that two probiotic strains, *Lactobacillus plantarum* and *L. curvatus* HY7601 and HY7601, respectively, showed high fructose-induced metabolic syndrome over a relatively short period, without any adverse effects. In contrast, probiotic treatment at 10^7 cfu/d modulated lipogenesis and β-oxidation related genes.

**Innovations and breakthroughs**

The authors showed that two probiotic strains, *Lactobacillus curvatus* (L. curvatus) HY7601 and *Lactobacillus plantarum* (L. plantarum) KY1032, can suppress metabolic abnormalities such as hypertriglyceridemia, hyperglycaemia and insulin resistance in high-fructose-induced metabolic syndrome. These probiotic health benefits were associated with decreased lipogenesis and increased β-oxidation-related gene expression in the liver.

**Applications**

Probiotic with *L. curvatus* HY7601 and *L. plantarum* KY1032 may provide a natural supplement for the management of the underlying risk factors of metabolic syndrome.
This paper investigated the effect of novel probiotics on the clinical characteristics of high-fructose-induced metabolic syndrome. The authors were suggesting a mechanism. One novel thing of this paper is that no one has looked at these specific microbes in this combination. It is well written.

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