Probiotic (yogurt) containing *Lactobacillus gasseri* OLL2716 is effective for preventing *Candida albicans*-induced mucosal inflammation and proliferation in the forestomach of diabetic rats

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**Summary.** Oral and esophageal candidiasis sometimes leads to mucosal hyperplasia, and progresses to carcinoma. We have produced an animal model for hyperplastic mucosal candidiasis in the forestomach that has a proliferative lesion of the squamous epithelium with chronic inflammation and *C. albicans* infection, some of which advanced to squamous cell carcinoma. There are many reports of the antibacterial effects of probiotics, but consensus about their antifungal effect has not been reached. In the present study, we investigate whether probiotic (yogurt) containing *Lactobacillus gasseri* OLL2716 (LG21 yogurt) can prevent proliferative and inflammatory changes caused by *C. albicans* in this mucosal candidiasis animal model. Diabetes was induced in 8-week-old WBN/Kob rats by intravenous administration of alloxan. One group of diabetic rats received a saline containing *C. albicans* and LG21 yogurt orally (DC+LG21 group) for 30 weeks, and another group received only *C. albicans* (DC group) for 30 weeks. They were sacrificed at 40 weeks of age, and analyzed histopathologically. In the DC+LG21 group, squamous hyperplasia at the greater curvature was significantly milder, and the Ki-67 positive index was significantly lower compared with the DC group. Suppurative inflammation with *C. albicans* also tended to be suppressed at the greater curvature. These findings suggest that probiotic (yogurt) containing *Lactobacillus gasseri* OLL2716 can suppress squamous hyperplastic change and inflammation associated with *C. albicans* infection in the forestomach.

**Key words:** Probiotics, *Candida albicans*, Inflammation, Diabetes, Squamous cell hyperplasia

**Introduction**

*Candida albicans* (*C. albicans*) is known to induce oral, esophageal and vaginal candidiasis (Bartholomew et al., 1987; Sitheeque and Samaranayake, 2003). Mucosal epithelia of candidiasis are thickened, and sometimes progress to carcinoma in patients infected with *C. albicans* (Gall et al., 2013; Norgaard et al., 2013). *C. albicans* in vitro is able to produce carcinogens such as nitrosamines (O’Grady and Reade, 1992; Williams et al., 2001; Dwivedi et al., 2009). In human, the oral mucosa of hyperplastic candidiasis is known to become dysplasia from hyperplasia, and to ultimately progress to carcinoma (Barrett et al., 1998; Williams et al., 2001; McCullough et al., 2002; Sitheeque and Samaranayake, 2003; Bakri et al., 2010). Therefore, infection with *C. albicans* is a critical factor in tumor development as well as a cause of inflammation.

Various experimental oral and/or gastrointestinal candidiasis are induced by the administration of immunosuppressive agents or antibiotics, but *C. albicans* infection persists for only a short time (Samaranayake and Samaranayake, 2001; Abe, 2004; Ishibashi et al., 2007; Costa et al., 2013). The proliferative candidiasis
that develop squamous dysplasia or carcinoma were induced by carcinogens such as 4-nitroquinoline-1-oxide and C. albicans for 32 weeks (O’Grady and Reade, 1992; Dwivedi et al., 2009). Without carcinogen treatment, oral epithelial hyperplasia and atypical epithelium have been found in rat affected with candidiasis for 12 months (Russell and Jones, 1975). That is, there is no model for proliferative candidiasis in which those persist for a long time and mucosal lesions with fungus and bacterial infections in the forestomach and that these lesions progressed to SCC (Kodama et al., 2006). We also demonstrated that C. albicans is greatly involved in this pathogenesis, by clarifying what lesions were suppressed by antifungal treatment and blood glucose control, and exacerbated by antimicrobial treatment (Kodama et al., 2006; Sano et al., 2009a,c). Additionally, we have succeeded in inducing early onset proliferative and inflammatory lesions by dosing C. albicans for diabetic rats, and defined a diabetic rodent model of C. albicans-induced mucosal inflammation and proliferation (Sano et al., 2014).

Probiotics are microorganisms, such as Lactobacillus, which have beneficial effects for the human body. They are useful for improving gastrointestinal disease and inflammatory bowel disease, and have antibacterial effects against harmful bacteria, such as Helicobacter pylori (Sakamoto et al., 2001; Uchida and Kurakazu, 2004; Uchida et al., 2010). Antifungal effects remain a highly controversial issue at present. Lactobacillus blocks C. albicans adhering to epithelial cells and inhibits fungal growth in vitro (Osset et al., 2001; Ishijima et al., 2012). Lactobacillus is also able to improve murine oral, gastric and vaginal candidiasis by inhibition of C. albicans growth and NF-κB activation (Hamad et al., 2006; Joo et al., 2012; Wagner and Johnson, 2012; Hayama et al., 2014). In human, Lactobacillus can reduce the Candida counts in oral and vaginal candidiasis (Falagas et al., 2006; Kraft-Bodi et al., 2015). On the other hand, some clinical trials do not support the effectiveness of Lactobacillus administered either orally or intravaginally on preventing colonization and infection by C. albicans in the vagina (Shalev et al., 1996; Pirotta et al., 2004; Falagas et al., 2006). Thus, clear evidence of the antifungal effect of Lactobacillus has not yet been obtained. In this study, we investigated whether a probiotic (yogurt) containing Lactobacillus gasseri OLL2716 is effective for preventing proliferative and inflammatory changes caused by C. albicans in alloxan-induced diabetic rats.

Materials and methods

Animals and diet

Female WBN/Kob rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). They were reared in a barrier-sustained animal room maintained at a temperature of 24±2°C with a relative humidity of 60±20%, and a 12 h light/dark cycle, and ventilated at least 12 times/h with sterilized fresh air. All rats were housed and reared in aluminum mesh cages. To protect against infection, the cages were changed once or more each week. Rats were given a pelleted diet (CRF-1; Oriental Yeast, Tokyo, Japan) and chlorinated water ad libitum. The study was approved by the Committee for Animal Experiments of Setsunan University.

Infection with Candida albicans

A strain of C. albicans, obtained from a rat forestomach with proliferative change in our previous study, was used for the inoculations in the present study. This strain was identified to be a close relative of Candida albicans ATCC18804 by gene analysis. The cultures were stored at -80°C in Microbank (pro-Lab Diagnostics, Ontario, Canada). A slant of potato dextrose agar was streaked with the organism 72 h before inoculation and allowed to incubate at room temperature (23°C). In many reports of experimental candidiasis, the most common dose range was 1x10⁶ to 10⁸ CFU/ml (Samaranayake and Samaranayake, 2001; Schofield et al., 2005; Hisajima et al., 2008; Dejima et al., 2011). The yeast cells were therefore rinsed from the slant with saline, and suspended at a concentration of approximately 6x10⁷ CFU/ml.

Experimental design

The experimental design was shown in Fig. 1. A total of 30 female WBN/Kob rats aged 8 weeks were given a single dose of alloxan (Sigma-Aldrich Japan, Tokyo, Japan) via the tail vein at a dosage of 40 mg/kg body weight (Sano et al., 2014). Concentrations were set at a given dose at which a rat could survive for a long period of time after developing signs of continuous hyperglycemia and glycosuria.

Diabetic rats were divided into 2 groups: the experimental group received oral administration of C.
*C. albicans* and a probiotic (yogurt) containing *Lactobacillus gasseri* OLL2716 (LG21 yogurt, Meiji Co., Ltd. Tokyo, Japan) (DC+LG21), and the control group received oral administration of only *C. albicans* (DC). In the DC group, 3 weeks after administration of alloxan, 1 ml of saline containing *C. albicans* (6x10^7 CFU/ml) was orally administered to each rat thrice a week for the initial two weeks. Thereafter, oral administration of *C. albicans* was carried out once a week until 32 weeks after administration of alloxan. In the DC+LG21 group, LG21 yogurt was orally administered to each rat (dose: 5 ml/kg body weight) once a day from 2 to 32 weeks after administration of alloxan, and 3 weeks after administration of alloxan, 1 ml of saline containing *C. albicans* (6x10^7 CFU/ml) was orally administered to each rat thrice a week for the initial two weeks. Thereafter, oral administration of *C. albicans* was carried out once a week until 32 weeks after administration of alloxan. At the end of the experiment (40 weeks of age), all rats in both groups were anesthetized with intraperitoneal injection of 40 mg/kg body weight ketamine hydrochloride (Ketalar, Sankyo, Tokyo) and 10 mg/kg body weight xylazine hydrochloride (Seractal, Bayer Japan, Tokyo) for histopathological examination.

The LG21 yogurt contained 1x10^7 cfu of *Lactobacillus gasseri* OLL2716/ml. Components contained in LG21 yogurt (112 g) were as follows: total energy, 89 kcal; protein, 3.8 g; lipid, 3.4 g; carbohydrate, 10.9 g; sodium, 49 mg; calcium, 134 mg.

**Glucosuria and glycem ia monitoring**

Fresh urine samples were collected in metabolism cages. Urinary glucose levels were measured semiquantitatively using a urine test paper (Wako Pure Chemical Industries, Osaka, Japan) every day from day 1 to day 3 after alloxan dosing, once every week for 1 month after the first week, and once every month thereafter from the fresh urine obtained from all alloxan-induced diabetic rats. Blood glucose levels were also measured semiquantitatively by the glucose oxidase method (Glutest E; Sanwa Kagaku, Aichi, Japan) once every month from the fourth week after dosing, using blood samples from the tail vein. Samples of fresh urine and blood from the tail vein were collected from 1:00 to 4:00 pm.

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![Fig. 2](image-url)

**Fig. 2.** Cutting line and normal structure of the stomach. **A.** The rat stomach was opened along the greater curvature, cut about 5 mm apart, and 6 pieces were obtained as the pictured 6 portions demarcated with red lines. Two middle pieces were selected and examined as lesser curvatures. Two pieces at both edges were selected and examined as greater curvatures. **B.** Macro-image of the stomach of health rat before fixation. **C.** Micro-image of the forestomach. HE stain. Scale bar: 100 μm.
**Histopathological analysis**

Moribund and dead animals (a total of 6 rats) of the DC and DC+LG21 groups were sacrificed and necropsied during the examination period; some of their organs were unavailable for histopathological examination because of cannibalism or autolysis. The remaining 24 rats were killed by exsanguination from the abdominal aorta under deep anesthesia at the end of each scheduled period. The entire alimentary tract was immediately removed following necropsy. The stomach was opened along the greater curvature and placed on cardboard (Fig. 2). It was cut sagittally about 5 mm apart, and 6 pieces were obtained. Two middle pieces were regarded as lesser curvature, and 2 pieces at both edges were considered as greater curvature. Organs of the 24 rats were immersed in 10% phosphate-buffered formalin solution immediately after necropsy. Fixed organs were trimmed, dehydrated by automated processor, and embedded in paraffin wax. Sections (4 µm thick) of tissue specimens were stained with hematoxylin–eosin and periodic acid-Schiff reaction (PAS) for histopathological examination.

Two investigators (KO and YT) evaluated and scored all lesions using our previous analytical criterion (Kodama et al., 2006; Sano et al., 2009a,c, 2014). Severity of proliferative lesions in the forestomach squamous epithelia was evaluated by the 5-grade grading system used in previous reports, i.e., Grade 0, slight change; Grade 1, mild change; Grade 2, moderate change; Grade 3, severe change; Grade 4, significantly severe change. Suppurative inflammation means neutrophil infiltration in the mucosal surface. Chronic inflammation means lymphocyte and plasma cell infiltration in the submucosa and lamina propria. The severity of suppurative inflammation and chronic inflammation was graded into 4 areas infiltrating the inflammatory cells of the mucosal surface, and submucosa and lamina propria, respectively; Grade 0, scarce (0%); Grade 1, focal change (<10%); Grade 2, multifocal changes (10-30%); and Grade 3, diffuse change (30-60%). The severity of *C. albicans* was graded into 3 grades as areas infiltrating *C. albicans* of the mucosal surface; Grade 0, scarce (0%); Grade 1, focal change (<10%); and Grade 2, multifocal changes (30-60%).

**Immunohistochemical analysis**

*C. albicans* infection was confirmed at the representative forestomach sections with the immunohistochemical technique. The cellular proliferation index was obtained from representative forestomach sections with immunohistochemical technique as well. The sections were deparaffinized in xylene and rehydrated through graded ethanol at room temperature. Rehydrated sections were digested by pepsin for 20 min at 37°C to retrieve antigen. Solutions and washes were prepared between the various steps using 0.05 M Tris buffer saline (TBS, pH 7.6) with 0.01% Tween 20. Non-specific endogenous peroxidase activity was blocked by exposure to 0.03% hydrogen peroxide in 100% methanol for 5 min, and masking was conducted with 5% normal goat serum in Tris-buffered saline for 5 min at room temperature. Incubation was carried out overnight at 4°C with anti *C. albicans* mouse monoclonal antibody (diluted 1:400, MAB806; Chemicon, USA) or anti Ki-67 rabbit monoclonal antibody (diluted 1:500, SP6; Epitomics, USA). Ki-67 is a cell cycle-related protein that is positive in the nucleus. Expression of Ki-67 antigen occurs preferentially during late G1, S, G2 and M phases of the cell cycle, but in cells in G0 phase this antigen cannot be detected. The slides were subsequently rinsed with TBS plus Tween 20, treated for 30 min at room temperature with Histofine simple stain rat MAX PO (M) (Nichirei, Tokyo, Japan) or Histofine simple stain rat MAX PO (Rb) (Nichirei, Tokyo, Japan), rinsed with TBS plus Tween 20, incubated in diaminobenzidine solution.
containing 0.01% hydrogen peroxide for the peroxidase coloring reaction, and counterstained with Mayer’s hematoxylin. Staining was negatively controlled by substituting mouse and rabbit isotype immunoglobulin diluted to the same concentration for the primary antibody. The Ki-67 positive index was estimated as a percentage of Ki-67-labeled nuclei/1000 squamous cells near the limiting ridge in the stomach.

Statistical analysis

The results obtained are expressed as the mean ± standard deviation (S.D.). Unpaired Student’s \( t \)-test was used for statistical analysis of the Ki-67 positive index, and Mann-Whitney \( U \) test was used to compare histopathological findings. When the calculated \( P \) value was less than 0.05, the difference was considered statistically significant. Statistical analysis was performed using the StatMate III program (ATMS, Tokyo, Japan).

Results

General conditions and monitoring of glucosuria and glycem ia

Two rats from the DC group and 4 from the DC+LG21 group died or were sacrificed between 12 and 37 weeks of age. The causes of death were attributed to malignant lymphoma (Sano et al., 2009b), urinary tract infection, ketoacidosis resulting from a severe diabetic condition, and technical dosing error.

In almost all rats, severe hyperglycemia (>300 mg/dl) and glucosuria (>500 mg/dl) continued for 32 weeks from the day of alloxan injection to the time of scheduled necropsy (DC: 12/13, DC+LG21: 10/11) (Fig. 3 A). One rat each in the DC and DC+LG21 groups showed normal to mild hyperglycemia and glucosuria, and they were excluded from the analysis because they did not satisfy the requirement for the defined animal model.

The mean body weights of all rats in the DC and DC+LG21 groups at necropsy were 183.2±22.4 g and 189.0±15.5 g, respectively. Increase in body weight did not differ between groups (Fig. 3 B).

Probiotic (yogurt) containing Lactobacillus gasseri OLL2716 suppresses C. albicans-induced mucosal proliferation

In both groups, squamous hyperplasia in the forestomach was mild at the greater curvature, but severe at the lesser curvature. In DC+LG21 group, squamous hyperplasia at the greater curvature was significantly milder (\( P<0.05 \)) than that in DC group (Fig. 4). Moderate (Grade 2) to severe (Grade 3) proliferative changes were detected in 8 out of 12 cases (66.7%) in the DC group, whereas moderate proliferative changes were observed in only 2 out of 10 cases (20%) in

Fig. 4. Probiotic (yogurt) containing Lactobacillus gasseri OLL2716 suppressed C. albicans-induced mucosal proliferation. Squamous cell hyperplasia of forestomach mucosa was milder in DC+LG21 group (A) than DC group (B). C. Severity of squamous cell hyperplasia in both groups. The bar indicates the mean value. * \( p<0.05 \) vs DC group. HE stain. Scale bar: 100 μm.
DC+LG21 group (Fig. 4). Ki-67 positive cells in both groups were mainly localized in the basal cells, with the Ki-67 positive index in the greater curvature of the DC+LG21 group (37.69±4.58%) being significantly lower (P<0.05) than that of the DC group (43.89±7.40%) (Fig. 5). At the lesser curvature, there were no clear differences in C. albicans-induced mucosal proliferation between groups.

**Effects of probiotic (yogurt) containing Lactobacillus gasseri OLL2716 on mucosal and submucosal inflammation induced by C. albicans**

In both groups, mucosal and submucosal inflammation with C. albicans in the forestomach was mild at the greater curvature, but severe at the lesser curvature. In the mucosal surface of squamous cell hyperplasia, neutrophils and macrophages with C. albicans and bacteria accumulated in both groups. Neutrophil infiltration of the mucosal surface (suppurative inflammation) was slightly suppressed at the greater curvature in the DC+LG21 group (P=0.127) compared to that in DC group (Fig. 6A-C). C. albicans was infected in the inflamed site, but there was no clear difference in either group (P=0.32) (Fig. 6D-F). At the lesser curvature, there was little difference in suppurative inflammation and C. albicans infection between the two groups.

In submucosa and lamina propria under squamous hyperplasia, many lymphocytes and plasma cells infiltrated (chronic inflammation). The severity and incidence of chronic inflammation were almost the same in DC+LG21 and DC groups.

**Discussion**

We demonstrated that a probiotic (yogurt) containing Lactobacillus gasseri OLL2716 suppresses C. albicans-induced mucosal proliferation. There is no definite evidence that C. albicans directly causes cancer development (Kuper et al., 2000; Coussens and Werb, 2002), but severe C. albicans infections in patients are often associated with neoplastic disease (Gall et al., 2013). In addition, oral mucosa of hyperplastic candidiasis is known to become dysplasia from hyperplasia, and to ultimately progress to carcinoma (Barrett et al., 1998; Williams et al., 2001; McCullough et al., 2002; Sitheeque and Samaranayake, 2003; Bakri et al., 2010). C. albicans induces SCC by directly producing carcinogenic constituents (Krogh et al., 1987; Hooper et al., 2009), and is a promoter of oral carcinogenesis in rat and mouse SCC models where carcinogenesis is initiated by 4-nitroquinoline-1-oxide (O’Grady and Reade, 1992; Williams et al., 2001; Dwivedi et al., 2009). Our previous reports indicated that C. albicans infections are deeply involved in mucosal proliferation and carcinogenesis in alloxan-induced diabetic rats, because these lesions are suppressed by antifungal treatment and enhanced by...
antibiotic treatment (Kodama et al., 2006; Sano et al., 2009c, 2012). In this study, a probiotic (yogurt) containing *Lactobacillus gasseri* OLL2716 inhibited *C. albicans*-induced mucosal proliferative change and decreased cell-proliferative activity in the forestomach. It is certain that a probiotic (yogurt) containing *Lactobacillus gasseri* OLL2716 plays an important role in inhibiting mucosal proliferation and carcinogenesis caused by *C. albicans* infection.

*Lactobacillus* reportedly blocks the adhesion of *C. albicans* to the vaginal epithelial cells *in vitro* (Osset et al., 2001; Ishijima et al., 2012); moreover, oral or vaginal candidiasis of murine models is suppressed by the oral or intravaginal administration of *Lactobacillus* (Hamad et al., 2006; Joo et al., 2012; Hayama et al., 2014). In human oral candidiasis, oral probiotics administration can reduce the prevalence of *Candida* (Kraft-Bodi et al., 2015). Meanwhile, in human vaginal candidiasis, oral or intravaginal probiotics administration has had contradictory results (Hilton et al., 1995; Reid et al., 2003; Falagas et al., 2006; Vicariotto et al., 2012), and clear evidence of the anti-inflammatory and antifungal effect of *Lactobacillus* has not been obtained yet. In this study, probiotics could not significantly reduce the extent of *C. albicans* infection, although they slightly suppressed *C. albicans*-induced suppurative inflammation. Thus, a probiotic (yogurt) containing *Lactobacillus gasseri* OLL2716 may have a weak anti-inflammatory effect against candidiasis.

*Lactobacillus* is considered to decrease the pH in the...
mucosa due to lactic acid production, and to acquire an antifungal effect (Sobel and Chaim, 1996; Antonio et al., 1999; Falagas et al., 2006). While many Lactobacillus were killed by gastric acid in the stomach, the gastric acid resistance of Lactobacillus gasseri OLL2716 was the highest among Lactobacillus bacteria (203 species) derived from human intestine (Kimura, 2004). Since a probiotic (yogurt) containing Lactobacillus gasseri OLL2716 easily colonizes in the gastric mucosa (Fujimura et al., 2006), the growth of C. albicans may be suppressed by the pH decrease due to lactic acid produced by Lactobacillus colonizing in the gastric mucosa. Probiotic (yogurt) containing Lactobacillus gasseri OLL2716 also demonstrates a protective effect on gastric mucosa by generating prostaglandin E2 (Uchida et al., 2010). In addition, Lactobacillus produces growth-inhibiting substances of bacteria and fungus, such as volatile fatty acid, hydrogen peroxide, acetaldehyde and bacteriocin (gassericin) (Piard and Desmazeaud, 1991; Kawai et al., 1998, 2004). Thus, the ability Lactobacillus gasseri OLL2716 has to colonize easily in gastric mucosa may have contributed to the antifungal and anti-inflammatory effects found in our study. In this case, there is no clear evidence that a probiotic (yogurt) containing Lactobacillus gasseri OLL2716 suppressed the growth of C. albicans directly, but it surely inhibited the mucosal proliferation and inflammation caused by C. albicans infection. Therefore, it is probable that a probiotic (yogurt) containing Lactobacillus gasseri OLL2716 may well suppress the growth of C. albicans.

In our previous studies, the epithelial proliferative lesion associated with C. albicans infection was severe in the lesser curvature compared with the greater curvature as well (Kodama et al., 2006). In the present study, a probiotic (yogurt) containing Lactobacillus gasseri OLL2716 was surely able to suppress mild proliferative lesion in the greater curvature, but there was no clear inhibitory effect on severe lesion of the lesser curvature. These results suggest that a probiotic (yogurt) containing Lactobacillus gasseri OLL2716 could suppress milder proliferative and inflammatory changes but not the severe ones. Therefore, probiotics may be used before the proliferative and inflammatory lesions are advanced.

It became clear that a probiotic (yogurt) containing Lactobacillus gasseri OLL2716 significantly suppresses proliferation of squamous epithelia cells with C. albicans and reduces suppurative inflammation caused by infection with C. albicans. We conclude that a probiotic (yogurt) containing Lactobacillus gasseri OLL2716 can be expected to become a prophylactic and/or therapeutic agent for mucosal proliferation and inflammation caused by Candida infection.

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