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Elimination Mechanism of *Candida albicans* in the Colon of BALB/c Mice by Dietary Fructo-oligosaccharide

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ABSTRACT

To test whether dietary fructo-oligosaccharides (FOS) eliminate *Candida albicans* from the gastrointestinal tract, BALB/c mice were inoculated intragastrically with *C. albicans* (1×10^7 cells/mouse) and then fed either a control diet or diet supplemented with a mixture of 1-kestose, nystose, and fructosylnystose, which is referred to as Meioligo-P, for four weeks. Recovery of organisms in the colon was significantly lower in mice fed Meioligo-P than in mice fed the control diet. This difference was abolished by ampicillin administration (1 mg/ml in drinking water). Meioligo-P increased total anaerobic and bifidobacteria attached to colon tissue and concentrations of short-chain fatty acids (SCFAs) in cecal contents. Although SCFAs suppressed hyphal formation of *C. albicans in vitro*, levels of hyphal formation of organisms cultured in the cecal contents of mice fed Meioligo-P did not differ from those of mice fed the control diet. These data suggest that FOS are prebiotics that reduce *C. albicans* in the colon. This action may be not attributed to SCFAs.

Keywords: *Candida albicans*, fructo-oligosaccharide, mice, short-chain fatty acids
Abbreviations: AD, atopic dermatitis; **CFU**, colony-forming unit; **FCS**, fetal calf serum; **FOS**, fructo-oligosaccharide; **SCFA**, short-chain fatty acid; **YPD**, yeast extract-peptone-dextrose

INTRODUCTION

Candida albicans is a member of the indigenous microbial flora of the gastrointestinal tract and mucocutaneous membranes in healthy humans. However, this diploid fungus has also the potential to act as a pathogen and is a frequent cause of complicating systemic infections and mortality in patients undergoing chemotherapy for cancer (Bodey 1984), immunosuppressive therapy (Myerowitz *et al.* 1977) or prolonged antibiotic therapy (Verghese *et al.* 1988). In addition, excessive colonization by *C. albicans* in the gastrointestinal tract may constitute an aggravating factor in atopic dermatitis (AD), although this remains controversial (Nikkels and Pierard 2003). As immunoglobulin E-mediated food allergy plays a pathogenic role in a subset of patients with AD (Eigenmann *et al.* 1998), gastrointestinal colonization by *C. albicans* may be involved in aggravation of AD by triggering a food allergy. We previously observed that gastrointestinal colonization by *C. albicans* promotes increases in serum antibodies specific to intragastrically administered ovalbumin in mice (Yamaguchi *et al.* 2006). This finding suggests that gastrointestinal colonization by *C. albicans* promotes sensitization against food antigens and increases the risk of food allergy. Elimination of *C. albicans* from the gastrointestinal tract could thus be a means of preventing not only systemic infection, but also food allergy and AD. Indeed, anti-fungal drugs improve the symptoms of AD (Back *et al.* 1995; Back and Bartosik 2001). However, anti-fungal drugs easily provoke the Jarisch-Herxheimer response, involving a transient exacerbation of symptoms soon after the first adequate dose of an appropriate antimicrobial drug used to treat an infection (Hurley 1995). Alternative treatments should thus be developed to safely and effectively eliminate *C. albicans* from the gastrointestinal tract.

Use of anti-bacterial antibiotics predisposes the individual to *C. albicans* infections, suggesting that host-associated bacteria play a role in controlling *C. albicans* populations (Wargo and Hogan 2006). Normal host-associated bacteria therefore appear to protect against *Candida* overgrowth. Use of probiotics and prebiotics to eliminate *C. albicans* from the gastrointestinal tract has thus gained a large amount of interest. Indeed, administration with *Lactobacillus* and *Bifidobacterium* species successfully protects against *Candida* colonization in the gastrointestinal tract of animals and humans (Wagner *et al.* 1997; Manzoni *et al.* 2006), As far as we know, however, the study by Buddington *et al.* (2002) is the only one investigating the effect of prebiotics on the colonization by *C. albicans*. They reported that the densities of *C. albicans* in the small intestine were lower in mice fed diets supplemented with oligofructose and inulin than in mice fed a diet supplemented with cellulose (Buddington *et al.* 2002).

Indigestible carbohydrates escape digestion and absorption in the small intestine and become available for colonic bacterial fermentation resulting in the production of short-chain fatty acids (SCFAs; acetic, butyric, and propionic acids) together with gases (CO_2, CH_4, and H_2) and heat (Topping and Clifton 2001). The fermentation product butyric acid reportedly suppresses serum-induced hyphal formation of *C. albicans in vitro* (Hoberg *et al.* 2001; Noverr and Huffnagle 2004). *C. albicans* can grow in a single-celled, budding yeast form or in a filamentous hyphal form, and the yeast-to-hyphal transition is an important feature of *C. albicans*, relevant to its colonization, invasion and pathogenesis in the host (Biswas *et al.* 2007). From these findings, we postulated that dietary indigestible oligosaccharides might eliminate *C. albicans* from the colon where the bacterial fermentation product butyric acid inhibits hyphal formation of the organisms. To test this hypothesis, the present study investigated the effect of dietary

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supplementation of a mixture of 1-kestose, nystose, and fructosylnystose, which is referred to as Meioligo-P, on gastrointestinal colonization by *C. albicans* using a recently established mouse model (Yamaguchi *et al.* 2005) and also examined the *in vitro* hyphal formation of *C. albicans*.

MATERIALS AND METHODS

Animals and diets

Specific pathogen-free female, 5-wk-old BALB/c mice (Japan SLC, Hamamatsu, Japan), were housed in a temperature-controlled (23 ± 2°C) room with a dark period from 20:00 to 08:00 h and *ad libitum* access to sterile water and a purified diet prepared according to AIN-93G (Reeves *et al.* 1993) criteria before the experiment. This diet was used as the control diet. A Meioligo-P-supplemented diet was prepared by adding 50 g/kg diet of Meioligo-P to the control diet in substitution to α-corn starch. Meioligo-P was donated by Meiji Food Materia (Tokyo, Japan). According to the manufacturer, Meioligo-P comprises D-glucose and D-fructose (1.3%), sucrose (2.5%), 1-kestose (37.3%), nystose (49.1%) and fructosylnystose (9.8%).

The present study was approved by the Hokkaido University Animal Use Committee, and mice were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Design for animal experiments

A total of 24 mice were divided into 2 groups (n = 12 each), and mice in one group were administered ampicillin sodium (1 mg/ml; Wako Pure Chemical Industries, Osaka, Japan) in drinking water throughout the experimental period. All mice were acclimatized to the control diet for 2 weeks, then inoculated intragastrically with *C. albicans*, as described below. One week after inoculation, mice of each group were further divided into 2 groups (n = 6 each) and fed either Meioligo-P-supplemented diet [(FOS (+)] or control diet [FOS (-)] for 4 weeks. The last day of the experiment, mice were anesthetized by diethyl ether and killed by exsanguination from the carotid artery. Following laparotomy, the stomach, jejunum, ileum and colon were excised, opened by longitudinal incision, washed with ice-cold sterile saline to remove the gross contents. Tissue samples were washed 3 times by gentle agitation in 5 ml of ice-cold sterile saline solution in a plastic centrifuge tube, then homogenized in 2 ml of ice-cold sterile saline solution. Each tissue homogenate was subjected to enumeration of *C. albicans* as described below. In the case of colon from mice without ampicillin administration, sterile anaerobic phosphate buffer was used for tissue washing and homogenization in order to count bacterial numbers as described below.

In a separate experiment, 12 mice were acclimatized to the control diet for 1 week. Mice were then divided into 2 groups (n = 6 each) and fed either FOS (-) or FOS (+) for 3 weeks without inoculation with *C. albicans.* On the last day of the experiment, mice were anesthetized by diethyl ether and killed by exsanguination from the carotid artery. Following laparotomy, the cecum was excised. Cecal contents were weighed and subjected to determination of SCFAs concentration and *in vitro* culture experiment, as described below.

Inoculation and enumeration of *C. albicans*

C. albicans (JCM 1542) was obtained from the Japan Collection of Microorganisms of The Institute of Physical and Chemical Research (Saitama, Japan) and maintained as previously described (Yamaguchi *et al.* 2005). For inoculation, mice were acclimatized to control diet for 2 weeks before being deprived of the diet for 16 h. Mice were then inoculated intragastrically with 0.2 ml of saline solution containing 1×10^7 cells/mouse of *C. albicans*. Tissue homogenates were quantitatively cultured using a standard pour plate technique as previously described (Yamaguchi *et al.* 2005).

Bacteriological analysis

Bacteriological analysis of colon tissue from mice was performed according to the methods described by Mitsuoka *et al.* (1965). Briefly, homogenates of colon tissue were diluted in 10-fold steps with anaerobic phosphate buffer, and a 50 µl sample of each dilution was then inoculated onto trypticase-soy-blood agar, glucose-blood-liver agar and modified lactobacillus selection agar for total aerobic, total anaerobic and lactobacilli, respectively. Numbers of bifidobacteria were counted using CPLX agar according to Yuki *et al.* (1999). Anaerobic incubation was performed at 37°C for 48 h using an AnaeroPack kit (Mitsubishi Gas Chemical, Tokyo, Japan), and aerobic incubation was performed at 37°C for 24 h. The number of colonies was counted after incubation and expressed as the logarithm of colony-forming units (CFU).

Measurement of SCFA concentrations in cecal contents

Concentrations of SCFAs in the cecal contents of mice were determined using HPLC by the internal standard method (Hoshi *et al.* 1994). A portion of cecal contents (approx. 100 mg) was homogenized in 2 ml of 10 mM sodium hydroxide solution containing 0.5 g/l crotonic acid (Wako Pure Chemical Industries) as an internal standard, then centrifuged at 10,000 × *g* for 15 min. Concentrations of individual SCFAs in supernatant were measured using an HPLC system (LC-6A, Shimadzu, Kyoto, Japan) equipped with a Shim-pack SCR-102H column (inner diameter, 8 mm; length, 30 cm; Shimadzu), column temperature 45°C, a mobile phase of 5 mM *p*-toluene sulfonic acid aqueous solution (flow rate 0.8 ml/min, 45°C), and a CDD-6A electroconductivity detector (Shimadzu).

In vitro culture experiments of *C. albicans*

C. albicans (initial concentration, 1×10^7 cells/ml) was cultured in yeast extract-peptone-dextrose (YPD) broth supplemented with different concentrations of each SCFA at 30°C with orbital shaking at 100 rpm for 24 h. The pH of the culture media was adjusted to 5.8. Growth in the yeast form of *C. albicans* was estimated by turbidimetry.

In a separate experiment, *C. albicans* (initial concentration, 1×10^7 cells/ml) was cultured in YPD broth supplemented with 10% fetal calf serum (FCS) and different concentrations (1, 2, 5, 10, 20, 50, and 100 mM) of each SCFA at 37°C with orbital shaking at 100 rpm for 4 h after culturing in FCS-free YPD broth at 30°C overnight. Hyphal formation was estimated by counting the number of organisms forming germ tubes under light microscopy (BX40; Olympus, Tokyo, Japan).

In addition, hyphal formation of organisms was examined in mouse cecal contents. A portion of cecal contents was homogenized in the same weight of PBS, then centrifuged at 10,000 × *g* for 10 min. The supernatant was then subjected to culturing organisms (initial concentration, 1×10^6 cells/ml). The pH of supernatant was not adjusted. Hyphal formation was estimated by counting the number of organisms forming germ tubes under light microscopy after culturing organisms in the supernatant at 37°C with orbital shaking at 100 rpm for 4 h.

Statistical analysis

Results are presented as mean ± SEM. To compare mean values, data were analyzed by an unpaired *t*-test or Tukey-Kramer's test following the F-test or two-way ANOVA. StatView for Macintosh version 5.0 software (SAS institute, Cary, NC, USA) was used for analysis. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Levels of colonization by *C. albicans* in the stomach of mice were the same in all groups (**Table 1**). FOS, ampicillin, and their interaction influenced colonization in the jejunum, whereas FOS did not influence colonization in the ileum. In the jejunum and ileum, levels of colonization were significantly higher in ampicillin-administered and FOS (+)-fed mice than in other animals. In the colon, FOS, ampicillin, and their interaction influenced colonization. Although

Table 1 Effect of dietary fructo-oligosaccharide and ampicillin in drinking water on colonization of *Candida albicans* in the gastrointestinal tract of BALB/c mice [1]

Fos/Amp [2]	Stomach	Jejunum	Ileum	Colon
Log10 CFU/g tissue				
-/-	5.26 ± 0.19 [a] (6/6)	3.23 ± 0.18 [a] (5/6)	3.68 ± 0.26 [a] (5/6)	3.16 ± 0.10 [a] (6/6)
+/-	5.10 ± 0.16 [a] (6/6)	3.09 ± 0.17 [a] (5/6)	3.22 ± 0.22 [a] (5/6)	< 2.4 (0/6)
-/+	5.15 ± 0.20 [a] (6/6)	3.69 ± 0.13 [a] (6/6)	3.48 ± 0.11 [a] (6/6)	4.26 ± 0.17 [b] (6/6)
+/+	4.94 ± 0.18 [a] (6/6)	4.58 ± 0.09 [b] (6/6)	4.62 ± 0.07 [b] (6/6)	4.58 ± 0.04 [b] (6/6)
ANOVA				
Fos	$P = 0.3271$	$P = 0.0156$	$P = 0.0606$	$P < 0.0001$
Amp	$P = 0.4714$	$P < 0.0001$	$P = 0.0024$	$P < 0.0001$
Fos × Amp	$P = 0.9042$	$P = 0.0017$	$P = 0.0002$	$P < 0.0001$

[1] Values represent mean ± SEM (n = 6). Mean values in a column with differing superscript letters are significantly different ($P < 0.05$). Values in parenthesis indicate the prevalence of colonization.
[2] Abbreviations: Amp, ampicillin; Fos, fructo-oligosaccharide; CFU, colony-forming units; n.d., not detectable.

Table 2 Effects of dietary fructo-oligosaccharide on numbers of commensal bacteria attached to colon tissue of BALB/c mice [1]

	FOS (-) [2]	FOS (+)
	Log10 CFU/g tissue	
Total aerobes	5.56 ± 0.15	5.58 ± 0.32
Total anaerobes	5.50 ± 0.28	6.72 ± 0.08*
Lactobacilli	4.67 ± 0.34	5.03 ± 0.36
Bifidobacteria	< 1.7	3.07 ± 0.24*

[1] Values represent mean ± SEM (n = 6).
[2] Abbreviations: FOS (-), control diet; FOS (+), diet supplemented with fructo-oligosaccharide; CFU, colony-forming units; n.d., not detectable.
* $P < 0.05$ vs. FOS (-).

Table 3 Effects of dietary fructo-oligosaccharide on concentrations of short-chain fatty acids in cecal contents of BALB/c mice [1]

	FOS (-) [2]	FOS (+)
	μmol/g contents	
Acetic acid	96.39 ± 4.34	85.65 ± 3.60
Propionic acid	14.17 ± 0.74	31.29 ± 3.59*
n-Butyric acid	10.23 ± 1.44	23.34 ± 1.70*

[1] Values represent mean ± SEM (n = 6).
[2] Abbreviations used: FOS (-), control diet; FOS (+), diet supplemented with fructo-oligosaccharide.
* $P < 0.05$ vs. FOS (-).

positive cultures were detected in the colons of untreated mice fed FOS (-) and ampicillin-administered mice, levels of colonization in the colons of untreated mice fed FOS (+) were under the detection limit. Levels of colonization in the colon were significantly higher in ampicillin-administered mice than in untreated mice. No organisms were found in feces or tissues of mice without inoculation of *C. albicans* (data not shown).

Although number of total anaerobic bacteria was significantly higher in mice fed FOS (+) than in those fed FOS (-), number of total aerobic and lactobacilli were the same for the two groups (**Table 2**). Mice fed FOS (+) displayed culturable bifidobacteria in the colon, whereas number of bifidobacteria in the colon of mice fed FOS (-) was under the detection limit. Bacteriological analysis was not performed in ampicillin-administered mice in the present study. We observed no detectable lactobacilli or bifidobacteria in colon tissues of ampicillin-administered mice in our preliminary experiments (unpublished data).

Table 3 shows concentrations of SCFAs in cecal contents of mice fed FOS (-) and FOS (+). Acetic acid was the major SCFA in cecal contents in both groups, and concentrations were the same between groups. In contrast, concentrations of propionic and *n*-butyric acids were significantly higher in mice fed FOS (+) than in mice fed FOS (-).

In vitro culture experiments showed that acetic, propionic and butyric acids significantly suppressed yeast growth at levels above 100, 50 and 5 mM, respectively (**Fig. 1A**). In addition, SCFA suppressed serum-induced hyphal formation in a dose-dependent manner (**Fig. 1B**), but minimum effective concentrations were lower for hyphal formation than for yeast growth. Acetic, propionic and butyric acids

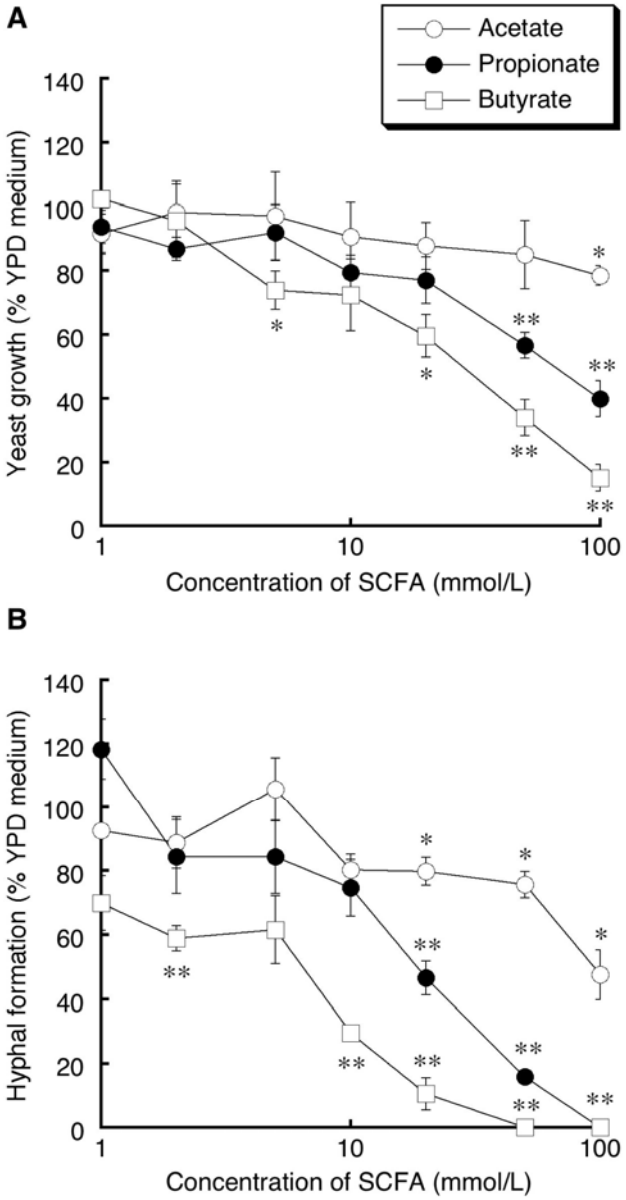

Fig. 1 Effects of SCFA on yeast growth (Chart A) and hyphal formation (Chart B) of *Candida albicans in vitro*. Values for organisms cultured with different concentrations of SCFA are expressed relative to the mean value for organisms cultured without SCFA, which is set to 100. Values represent mean ± SEM of 3 independent experiments. *$P < 0.05$ and **$P < 0.01$ vs. YPD broth without SCFA.

significantly suppressed hyphal formation at levels above 20, 20 and 2 mM, respectively. Hyphal formation was completely inhibited by 50 mM butyric acid.

Although no hyphal formation was observed in organisms cultured in PBS, cecal supernatants stimulated hyphal

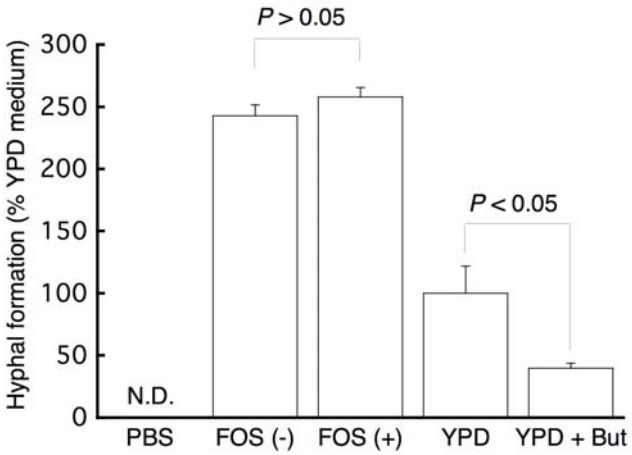

Fig. 2 Hyphal formation of *Candida albicans* in the cecal contents of BALB/c mice *in vitro*. Values are expressed relative to the mean value for organisms cultured in serum-supplemented YPD broth, which is set to 100. Values for FOS (-) and FOS (+) represent mean ± SEM of 6 mice, while values for PBS, YPD broth and YPD broth plus 10 mM butyric acid represent mean ± SEM of triplicate measurements. N.D., not detectable.

formation (**Fig. 2**). Levels of hyphal formation were the same between organisms cultured in cecal supernatant from mice fed FOS (-) and FOS (+). Supplementation of 10 mM butyric acid to YPD broth significantly suppressed serum-induced hyphal formation.

DISCUSSION

The present study clearly demonstrated that dietary FOS reduce *C. albicans* in the colon of mice, but not in the stomach or small intestine. In addition, we confirmed that dietary FOS caused proliferation of anaerobic bacteria including bifidobacteria in the colon, being in agreement with the previous studies (Mitsuoka *et al.* 1987; Hidaka *et al.* 1991; Bouhnik *et al.* 1996). Furthermore, reduction of *C. albicans* in the colon by dietary FOS was completely abolished by anti-bacterial antibiotic treatment. Given that, host-associated bacteria may play a role in controlling *C. albicans* populations (Wargo and Hogan 2006), our observations suggest that reduction of *C. albicans* in the colon by dietary FOS is attributed to the increase in the bacterial populations of the colon.

Commensal bacteria are considered to control *Candida* colonization in the gastrointestinal tract through different mechanisms such as competition for nutrients, steric exclusion for the adhesion to the epithelium, stimulation of the host's immune system and production of compounds affecting epithelial adherence, yeast growth and hyphal formation of *C. albicans* (Wargo and Hogan 2006). Butyric acid, a principal fermentation product of commensal bacteria, reportedly inhibits hyphal formation of *C. albicans in vitro* (Hoberg *et al.* 2001; Noverr and Huffnagle 2004). Because hyphal formation of *C. albicans* is important for its colonization, invasion and pathogenesis in the host (Biswas *et al.* 2007), it is naturally possible to claim that butyric acid is a compound controlling *Candida* colonization *in vivo.* In this regard, *in vitro* culture experiments in the present study confirmed that not only butyric acid but also acetic and propionic acids suppress growth in the yeast form and serum-induced hyphal formation of *C. albicans* in a dose-dependent manner. As concentrations of each SCFA used in the present study were within the range observed *in vivo* (Cummings and Macfarlane 1991), the suppressive effects of SCFA on *C. albicans* seem physiologically relevant. In addition, minimum effective concentrations of each SCFA were lower for hyphal formation than for yeast growth, suggesting that the inhibition of hyphal formation is not due to the inhibition of yeast growth by its antifungal activity.

Such *in vitro* findings naturally led us speculate that increased production of SCFAs by bacterial fermentation would be responsible for reduction of *C. albicans* by dietary FOS *in vivo*. Indeed, concentrations of propionic and butyric acids in cecal contents were significantly higher in mice fed FOS (+) than in mice fed FOS (-), supporting previous reports (Campbell *et al.* 1997; Le Blay *et al.* 1999). To further examine our idea, we compared hyphal formation of *C. albicans* cultured in the supernatant of cecal contents from mice fed FOS (-) or FOS (+). Contrary to our expectations, cecal contents from mice fed FOS (+) failed to reduce hyphal formation of *C. albicans* as compared to those from mice fed FOS (-). Thus, hyphal formation may not be reduced in spite of higher concentrations of SCFAs in the colon of mice fed FOS (+). Supplementation of serum to culture media is usually required for hyphal formation of *C. albicans in vitro*. The fact that hyphal formation occurred in the supernatant of cecal contents without serum supplementation in the present study is therefore noteworthy. The ability to induce hyphal formation was higher in the supernatant of cecal contents than in YPD broth supplemented with 10% serum. The results suggest that cecal contents contain certain substances stimulating hyphal formation of *C. albicans*. These substances may overwhelm the inhibitory action of SCFAs on hyphal formation of *C. albicans*. It remains unclear whether these substances are derived from host, indigenous bacteria or dietary components. Identification of such substances may lead to the discovery of novel targets to inhibit colonization and dissemination by *C. albicans*.

Nevertheless, SCFAs may indirectly affect *Candida* colonization in the colon. Mucins, i.e., mucus *O*-linked glycoproteins, are produced and secreted by goblet cells in the gastrointestinal tract, forming a gel that plays an important role in lubrication and protection of mucosal surfaces (Forstner and Forstner 1994). de Repentigny *et al.* (2000) demonstrated that mucin purified from rabbit intestine blocks adhesion of *C. albicans* to human buccal epithelial cells *in vitro.* In addition, Ogasawara *et al.* (2007) showed that salivary mucin inhibits hyphal formation of *C. albicans in vitro* (Ogasawara *et al.* 2007). Furthermore, we previously showed that dietary FOS increase mucin contents in the cecum of rats (Tanabe *et al.* 2006). Because mucin production and secretion are stimulated by SCFAs in the colon (Barcelo *et al.* 2000; Shimotoyodome *et al.* 2000), increased SCFAs in the colon of mice fed FOS may contribute to the reduction of *C. albicans* through mucin production and secretion.

In conclusion, we propose that FOS are a prebiotic that reduce the opportunistic pathogen, *C. albicans*, in the colon, but this action might not be attributed to inhibition of hyphal formation by SCFAs. In humans whose colon is colonized by *C. albicans*, consumption of indigestible oligosaccharides such as FOS may reduce the risk for systemic candidiasis and allergic diseases. The present study therefore provides additional information on the beneficial effect of dietary indigestible oligosaccharides for human health.

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