Dietary Oligofructose and Inulin Protect Mice from Enteric and Systemic Pathogens and Tumor Inducers[1]

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ABSTRACT Prebiotics induce changes in the population and metabolic characteristics of the gastrointestinal bacteria, modulate enteric and systemic immune functions, and provide laboratory rodents with resistance to carcinogens that promote colorectal cancer. There is less known about protection from other challenges. Therefore, mice of the B6C3F1 strain were fed for 6 wk a control diet with 100 g/kg cellulose or one of two experimental diets with the cellulose replaced entirely by the nondigestible oligosaccharides (NDO) oligofructose and inulin. From each diet, 25 mice were challenged by a promoter of colorectal cancer (1,2-dimethylhydrazine), B16F10 tumor cells, the enteric pathogen *Candida albicans* (enterically), or were infected systemically with *Listeria monocytogenes* or *Salmonella typhimurium*. The incidences of aberrant crypt foci in the distal colon after exposure to dimethylhdrazine for mice fed inulin (53%) and oligofructose (54%) were lower than in control mice (76%; $P < 0.05$), but the fructans did not reduce the incidence of lung tumors after injection of the B16F10 tumor cells. Mice fed the diets with fructans had 50% lower densities of *C. albicans* in the small intestine ($P < 0.05$). A systemic infection with *L. monocytogenes* caused nearly 30% mortality among control mice, but none of the mice fed inulin died, with survival intermediate for mice fed oligofructose. Mortality was higher for the systemic infection of *S. typhimurium* (>80% for control mice), but fewer of the mice fed inulin died (60%; $P < 0.05$), with mice fed oligofructose again intermediate. The mechanistic basis for the increased resistance provided by dietary NDO was not elucidated, but the findings are consistent with enhanced immune functions in response to changes in the composition and metabolic characteristics of the bacteria resident in the gastrointestinal tract. J. Nutr. 132: 472–477, 2002.

KEY WORDS: • *nondigestible oligosaccharides* • *Salmonella typhimurium* • *Listeria monocytogenes* • *Candida albicans* • *dimethylhydrazine* • *Mus musculus*

The interactions among dietary inputs, immune functions, and protection against pathogens and other health challenges are complex. For example, adequate intakes of energy and nutrients are critical for normal immune functions (1), but chronic consumption of diets high in fat and protein of animal origin is associated with an increased incidence of colorectal cancers (2). Supplementing the diet with fiber-rich foods has been associated with a lower risk of developing cancer (3). It is considered important for maintaining barrier functions of the gastrointestinal tract (GIT)[3] mucosa and has been shown to decrease the risk of bacterial translocation and septicemia (4–6). However, not all sources of fiber appear to provide the same degree of immunomodulation (7) and therefore health benefits.

The bacterial populations resident throughout the GIT are important mediators of the influence of dietary inputs on immune functions (8) and themselves are a critical determinant of disease resistance (9,10). Changes in the bacterial assemblages resident in the GIT, whether induced by diet, administration of antibiotics or by other means, can influence the functions of the enteric and systemic components of the immune system (10). Although much more is known concerning the interactions between the pathogenic bacteria and immune functions, there is increasing awareness that certain groups of bacteria can enhance resistance and stimulate defense mechanisms. Of particular interest are the lactic acid–producing bacteria (LAB), which inhibit the growth of pathogens and other bacterial groups considered to be detrimental (11), stimulate immune functions (12), and reduce the incidence and severity of tumor development in animals challenged with carcinogens and tumor cells (13).

The LAB are a diverse, heterogeneous group of bacteria that normally represent <1% of the fecal flora of adult humans (14). Representative genera include the *Lactobacilli*, *Bifidobacteria*, *Streptococci*, among others (15), all of which produce lactate as the principal product of fermentation (16). Densities of LAB can be increased by supplementing the diet with either viable LAB (probiotics) or substrates that selectively encourage the growth of LAB (prebiotics). The majority of prebiotics are nondigestible oligosaccharides, with the β-fructans, inulin and oligofructose, the most commonly studied (11).

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[3] Abbreviations used: ACF, aberrant crypt foci; cfu, colony forming units; DMH, 1,2-dimethylhydrazine;GIT, gastrointestinal tract; LAB, lactic acid–producing bacteria; NDO, nondigestible oligosaccharides.

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The present paper reports the efficacy of inulin and oligofructose at enhancing enteric and systemic protection against challenges by three pathogens (two systemic and one enteric), a carcinogen that induces aberrant crypt foci in the colon and a cell line that induces lung tumors. Concurrent studies using the same mice (B6C3F1 strain) verified that diets containing 100 g/kg inulin or oligofructose increase the densities of LAB (17) and modulate some immune functions (unpublished data). Unlike the short or no prefeeding periods used in the majority of challenge studies, the diets were fed for a prolonged period (6 wk) before the challenges. This period was considered sufficiently long to allow for full adaptation of the GIT ecosystem and extends past the possible transient influences of nondigestible oligosaccharides (NDO) on the composition and metabolic characteristics of the resident bacteria (18).

MATERIALS AND METHODS

Mice and their care

All phases of the research involving the use of animals were approved by the Mississippi State University Institutional Animal Care and Use Committee and were performed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. Female mice of the B6C3F1 strain were obtained at 32–35 d of age and from the same supplier (Charles Rivers Laboratories, Wilmington, MA) and the same production site. The mice were distributed in groups of 5 to cages that were located in a room maintained at 22°C with a 12-h light:dark cycle.

The groups of mice were assigned to three diets that were based on the AIN 76A rodent diet (19) with 100 g/kg fiber. The control diet had cellulose as the sole source of fiber and the two experimental diets had the cellulose replaced entirely by oligofructose (Raftilose P95; Orafti, Tienen, Belgium) or inulin (Raftiline HP; Orafti, Tienen, Belgium). The diets were fed to excess as pellets for 6 wk before and during the pathogen and cancer challenges. Consumption of each diet was recorded for 5 groups of mice (25 mice) during a 45-d period. Water was continuously available.

Challenge protocols

For each of the following challenge protocols, variation was minimized by obtaining all of the mice to be used in a single shipment from the same production facility. For each study, the shipment of mice was distributed to the three treatments (25 per treatment) and the mice were simultaneously exposed to the health challenge. Because it was not possible to perform all of the challenges concurrently, several shipments of mice were obtained over a 1-y period.

Enteric defense functions. The abilities of the mice to clear an intestinal pathogen and prevent its translocation outside of the GIT were assessed by oral administration of the yeast *Candida albicans* and enumeration of the resulting densities 7 d later in the contents of the mid-small intestine and the mesenteric lymph nodes of mice killed by CO_2 asphyxiation. The experimental protocol was established in a preliminary trial that exposed 40 mice of the same strain and fed a nonpurified diet (Lab Diet 5001 Rodent Chow, PMI Nutritionals, Brentwood, MO) to *C. albicans* (orally) that had been cultured in tryptic soy agar broth. Briefly, the contents of the mid-small intestine and mesenteric lymph nodes were collected after 1, 3, 5 and 7 d (n = 10 mice at each day) and homogenates were prepared from each sample using tryptic soy agar broth. Serial dilutions were plated on BIGGY agar (BBL, Becton-Dickinson, Cockeysville, MD) and the number of colony forming units (cfu) was enumerated after incubating for 24–48 h at 37°C. *C. albicans* was detected in the small intestine contents at all time points. In contrast, after 1, 3 and 5 d, the mesenteric lymph nodes of only three mice were colonized, and at very low densities. At 7 d, significant densities ($>10^3$ cfu) were recovered from the mesenteric lymph nodes of 3 of 10 mice. *C. albicans* was not detected in the contents of the small intestine and mesenteric lymph nodes of 10 unexposed mice that were examined at the same time as the group exposed for 7 d. On the basis of results from the preliminary trial, mice fed the control and the two experimental diets for 6 wk (25 per diet) were examined 7 d after being inoculated orally with *C. albicans*.

The responses of the GIT to a carcinogen were studied by exposing 25 mice from each treatment to the carcinogen 1,2-dimethylhydrazine (DMH), which induces the appearance of aberrant crypt foci (ACF) in the colon, particularly the distal half (20). ACF are commonly considered indicators of early, preneoplastic lesions in the colon and as precursors for colon cancer (21), but not all ACF develop into tumors (22). Mice were injected subcutaneously with DMH (20 mg/kg body mass) once per week for 6 wk. The mice were killed 4 wk after the last DMH injection and the entire colon was fixed in neutral buffered formalin and later stained with hematoxylin. The numbers of ACF in the proximal, mid- and distal thirds of the colon were recorded. Lymphoid follicles have been implicated in having a promotional role after initiation of carcinogenesis (23) and were counted in each region.

Systemic defense functions. The ability to defend against systemic infections was assessed by recording survival 2 wk after intraperitoneal injection with the pathogens *Listeria monocytogenes* and *Salmonella typhimurium*. Both pathogens were passaged in mice of the same strain three times to increase virulence, after which preliminary studies were conducted with each pathogen to identify doses that would be suitable for determining whether the different treatments influence survival. For the preliminary studies, B6C3F1 mice of the same age as the experimental mice were infected intraperitoneally with 7 densities of each pathogen ranging from 10^2 to 10^8 cfu (0.1 mL of 10^6 to 10^{12} cfu/L; n = 10 mice for each dose). Mortality was recorded twice daily for 2 wk. The challenge studies showed that 5×10^6 *L. moncytogenes* would cause 30–40% mortality. The B6C3F1 mice were less resistant to *S. typhimurium* and a dose of 10^3 cfu resulted in 70–80% mortality. These infective doses were selected for the experimental challenges.

L. monocytogenes of the virulent EGD strain (24) were grown on blood agar plates at 37°C for 24 h. The bacteria were harvested, suspended in a 9 g/L saline solution and washed twice by centrifugation (3200 × *g*; 5 min). The sedimented bacteria were suspended in a sterile solution of 9 g/L NaCl and propagated overnight at 37°C in tryptose broth that was shaken. The suspension of growing bacteria was diluted to an optical density that corresponded to a desired infective dose of $1–5 \times 10^{10}$ cfu/L, which was confirmed by plating on blood agar plates. The *L. monocytogenes* was given to 25 mice by an intraperitoneal injection of 0.1 mL.

S. typhimurium (ATCC strain 14024) were cultivated from the spleens of B6F3F1 mice that had been infected and died. The virulent *S. typhimurium* were grown on blood agar plates at 37°C for 24 h. The bacteria were harvested, suspended in 9 g/L saline and washed twice by centrifugation (3200 × *g*; 5 min). The sedimented bacteria were suspended in a sterile solution of 9 g/L NaCl to an optical density that corresponded with the desired concentration of $\sim 10^7$ bacteria/L. Actual densities were enumerated on blood agar plates. *S. typhimurium* was given to 25 mice by injecting 0.1 mL of the suspension intraperitoneally.

Tumor cell challenges are often used as another means to assess host resistance (25). To do so, another 25 mice from each diet group were injected subcutaneously with 5×10^4 B16F10 tumor cells that had been prepared from tumor nodules that had been induced in 3 mice. This number of tumor cells was shown to result in about 50% tumor incidence in B6C3F1 mice of comparable age. The number of nodules on the surface of the lungs was recorded 28 d after administering the tumor cells.

Statistical analyses

Values in the figures are means and SEM. The PROC General Linear Models procedure of the Statistical Analysis System (SAS Institute, Version 7.0, Cary, NC) was used to identify an effect of treatment (diet) on the measured variables. For the *Listeria* and *Salmonella* challenges, the percentage of mice surviving was calculated for each cage with each cage used as a single measurement. For the other measurements (i.e., number of ACF in the colon after DMH exposure, densities of *Candida*, number of lung tumors after

injection of B16F10 tumor cells), the individual mice were considered as sample units. Specific differences between treatments were identified using Duncan's test and paired *t* tests, with $P < 0.05$ recognized as the critical value for significant difference.

RESULTS

Food consumption. The amount of food consumed during the 30-d period varied significantly among mice fed the three diets ($P < 0.05$ for all comparisons), with the cellulose diet consumed the most [2.32 ± 0.04 g/(mouse · d), the oligofructose diet the least (2.10 ± 0.02) and the inulin diet intermediate (2.20 ± 0.02).

Candida albicans challenge. Densities of *C. albicans* in the contents of the small intestine did not differ between the mice fed the experimental diets with oligofructose and inulin, but both were lower than values for control mice (**Fig. 1**). Corresponding to the preliminary study, *C. albicans* was not found consistently in the mesenteric lymph nodes (9/45 mice) and differences were not detected among groups for the incidence and densities. When present, densities were generally <100 cfu/g (the mesenteric lymph nodes of only 1 of the 75 mice had >100 cfu).

DMH challenge. There was a significant increasing proximal to distal gradient for the incidence of ACF in the colon (**Fig. 2**, *lower panel*). The number of ACF in the proximal colon was low and virtually identical among the control and experimental diet groups (Fig. 2, *upper panel*). However, mice fed the oligofructose and inulin diets had incidences of ACF in the mid- (46 and 42%, respectively) and distal (54 and 53%, respectively) colon that were lower than in the same regions of mice fed the control diet (72% and 76%, respectively), $P < 0.05$.

The total number of ACF (sum of single and multiple foci) in the entire colons of mice fed the control diet (8.2 ± 1.5) was greater than in those fed inulin (4.6 ± 1.3; $P < 0.05$), and tended to be greater mice fed the diet with oligofructose (5.4 ± 1.6; $P = 0.08$). The differences among the groups were more pronounced in the distal colon where values for control mice exceeded those for mice fed the diet with oligofructose ($P < 0.05$) and tended to be greater than in those fed inulin ($P = 0.08$).

The total and regional densities of lymphoid follicles did not differ among mice fed the three diets.

Listeria monocytogenes challenge. The systemic challenge with the pathogenic strain of *L. monocytogenes* resulted in 28% mortality (± 5%) 14 d after infection when mice were fed the control diet (**Fig. 3**A). None of the mice fed inulin died. Mortality was intermediate for mice fed the diet with oligofructose, but still lower than that of the control mice ($P < 0.05$). The addition of oligofructose or inulin to the diet did not delay the onset of mortality or alter the pattern of decline in the number of surviving mice.

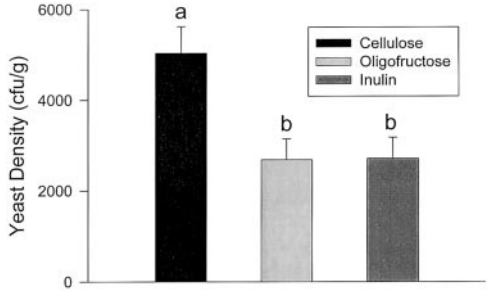

FIGURE 1 Densities of *Candida albicans* (cfu/g) in the contents of the small intestine 7 d after oral inoculation of mice that had been fed diets with 100 g/kg fiber from cellulose, oligofructose and inulin. Values are means ± SEM, $n = 25$; bars with different letters differ, $P < 0.05$.

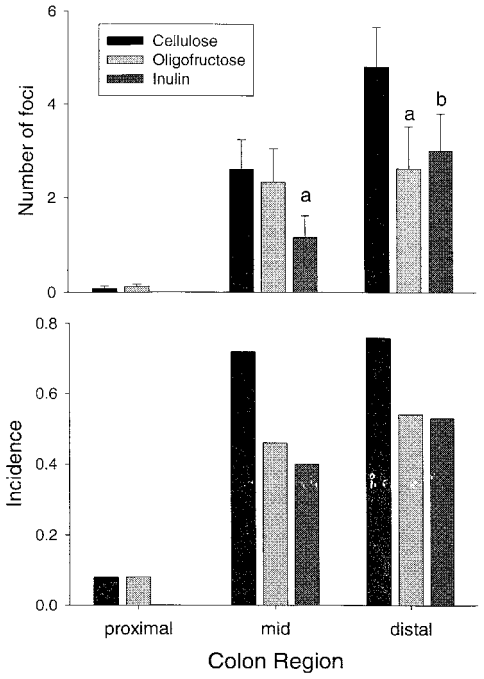

FIGURE 2 The number of aberrant crypt foci (*upper panel*) and the incidence of aberrant crypt foci (*lower panel*) in the proximal, mid-, and distal colons of mice fed diets with100 g/kg fiber from cellulose, oligofructose and inulin for 6 wk before a 6-wk exposure to the carcinogen 1,2-dimethylhydrazine. Values are means ± SEM, $n = 25$; bars labeled with the letter "a" differ from the cellulose treatment ($P < 0.05$) and the letter "b" denotes $0.05 < P < 0.10$.

Salmonella typhimurium challenge. Consistent with the preliminary study, mortality for all treatments was higher when mice were challenged with a lower infective dose of *S. typhimurium* compared with the dose of *L. monocytogenes* (Fig. 3B). Mice fed the diet with inulin had lower mortality compared with control mice ($P < 0.05$). Mortality for mice fed the diet with oligofructose was intermediate, but was not different from the control or inulin treatments. The onset and pattern of decline for mortality did not vary among treatments.

B16F10 tumor cell challenge. The incidence of tumors detected on the surface of the lungs of control mice (60%) did not differ from that of mice fed the diets with oligofructose (52%) or inulin (72%). The total number of tumors per mouse did not differ among mice fed the diets with cellulose (1.7 ± 0.2), oligofructose (1.7 ± 0.2) and inulin (1.6 ± 0.03).

DISCUSSION

Dietary inputs influence the immune system directly by providing energy and nutrients needed by the host (1) and indirectly by eliciting changes in the population and metabolic characteristics of GIT bacteria (8). Although fiber is considered to be an important component of a healthy diet, the present study indicates that not all fibers provide the same resistance to health challenges. Specifically, poorly fermented fibers, such as crystalline cellulose, do not selectively encourage the proliferation of LAB and are not as effective as the

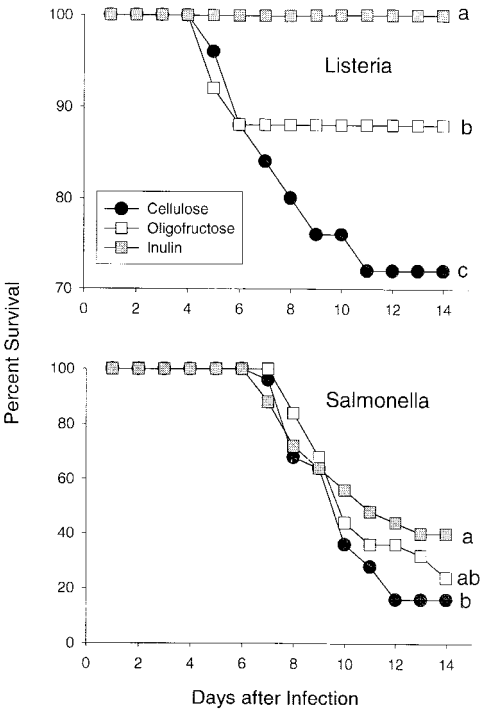

FIGURE 3 Mortality curves for mice fed diets with 100 g/kg fiber from cellulose, oligofructose and inulin for 6 wk before an intraperitoneal injection of *Listeria monocytogenes* (*upper panel*) and *Salmonella typhimurium* (*lower panel*). Values are means ± SEM, $n = 25$; curves not sharing a letter had different survivals 2 wk after the infection, $P < 0.05$.

β-fructans oligofructose and inulin at increasing resistance against some, but not all, health challenges.

There is no evidence to indicate that inulin or oligofructose directly inhibit the growth of *Candida* and other enteric pathogens or directly stimulate immune functions. The enhanced host resistance to pathogens has instead been attributed in part to the ability of β-fructans and other NDO to cause changes in the population and metabolic characteristics of the bacterial assemblages present in the GIT (26). The LAB are of particular interest from a health perspective because of their ability to inhibit the growth of other bacterial species, notably pathogens, by fermenting dietary inputs and reducing GIT pH and by secreting antimicrobial compounds (27). Correspondingly, the lower lumenal densities of *Candida* in mice fed the diets with oligofructose and inulin coincided with higher densities and proportions of LAB (17). Moreover, the LAB also stimulate numerous innate and acquired immune functions (10,28–30), possibly by modulating cytokine expression (31).

The similar low incidence and consistently low densities of C. *albicans* in the mesenteric lymph nodes among the treatments, despite the differences in lumenal densities, indicate that the mucosal barrier was sufficiently intact in all groups to provide some protection against translocation of C. *albicans*. The integrity of the mucosal barrier is a critical determinant of whether enteric pathogens are able to translocate and disseminate throughout the systemic circuit. Although the incidence of bacterial translocation can be reduced by promoting higher densities of LAB (32) or other beneficial organisms, such as *Saccharomyces boulardii* (33), dietary fiber, regardless of how well it is fermented, is sufficient to maintain the mucosal barrier (4–6). Because the control mice were fed a diet with 10% cellulose, it is not surprising that translocation in this group was minimal, despite the higher lumenal densities of *Candida*. The similar low incidence of translocation for the mice used in the preliminary study corresponds to the feeding of a commercial nonpurified diet that contains fiber (crude fiber reported as not >6%).

Some species of *Lactobacilli* stimulate immune functions and increase resistance to pathogens when administered as probiotics (34), injected intravenously (35,36) or given intraperitoneally (37). The present results provide the first evidence, to our knowledge, that resistance against systemic infections by pathogenic bacteria can also be increased by feeding diets that selectively encourage the proliferation of LAB already resident in the GIT. Although the mechanisms responsible for the heightened resistance to *Salmonella* and *Listeria* conferred by inulin and oligofructose were not determined, recovery from infections in mouse models is dependent in part on sensitization of T cells, which activate and enhance bactericidal activity of lymphocytes (25). The higher densities of LAB in the GIT of mice fed the β-fructan–containing diets (17) coincided with heightened sensitivity of lymphocytes, increased phagocytic activity of unactivated peritoneal macrophages, enhanced natural killer cell activity of splenocytes but not a redistribution of maturing lymphocytes (unpublished data).

The population and metabolic characteristics of the GIT bacteria are important determinants for the growth of transplanted tumor cells (38,39) and the carcinogenicity of various compounds used to experimentally induce ACF (40–43). Increasing the densities and proportions of LAB reduces the activities of enzymes implicated in carcinogenesis (44), lowers the concentrations of cecal ammonia (40) and decreases the incidence of tumors after exposure to known carcinogens (36). The lower incidence, number and size of ACF in mice fed diets with the two fructans corroborate the relationship among densities of LAB, conversion of DMH to a carcinogen and the occurrence of ACF in rats (13). However, interactions among dietary fiber, dose of carcinogen, and immune responses may complicate interpretations (45). The benefits of diets supplemented with β-fructans apply even to mice susceptible to spontaneous colon cancers (46). The improved resistance to tumor development conferred by higher densities of LAB has been attributed to heightened T-lymphocyte functions (29), enhanced macrophage activity (39) and modulation of other immune functions, including T lymphocytes (26). Supplementing the diet with NDO may provide another mechanism of resistance by increasing rates of apoptosis (47), apparently due to higher concentrations of butyrate (48) from fermentation of NDO (49,50). Although fermentation of β-fructans and other NDO by LAB increases the lumenal concentrations of short-chain fatty acids, the associated reduction in lumenal pH does not provide protection (51). Collectively, these findings validate the contention that β-fructans, and possibly other NDO, can be considered as antimutagens (52), and probably act by beneficially modulating the assemblages of bacteria in the GIT. However, the associated enhancement of some immune functions of B6C3F1 mice fed the same diets (unpublished data) did not reduce the incidence and number of lung tumors.

The higher incidence of ACF in the distal half of the colon after exposure to DMH agrees with previous studies (13) and has led some to consider the distal colon to be more sensitive to carcinogens (53). Alternatively, the beneficial influences associated with bacterial fermentation of dietary fibers may be more pronounced in more proximal regions (54). Correspondingly, the influences of NDO on the bacterial assemblages are more pronounced in the proximal than distal colon (55).

Although the total densities of LAB present in the GIT are of obvious importance (56), the magnitude of health benefits

is also related to the species of LAB that are present (34,36,42,57,58). The present study, like many others, used commercially available mice that are derived from an original stock of gnotobiotic animals that were inoculated with the "Altered Schaedler Flora" (59). This probiotic includes only a few representatives of the *Lactobacilli*, whereas *Bifidobacteria* and other members of the LAB are absent. Over time, additional species of bacteria colonize the GIT, leading to a more complex and diverse assemblage of species. The direct relationship between the increased densities of LAB and the improved resistance of the mice fed diets with inulin and oligofructose indicates that the bacteria that were resident in the GIT of the experimental mice were capable of providing health benefits. It is possible that the presence of a greater diversity of LAB would have increased the magnitude of the diet-induced stimulation of immune functions and the resulting resistance against health challenges.

There has been concern that the influences of NDO on the composition and metabolic characteristics of the resident bacteria may be transient (18) and may lead to diminished benefits, perhaps by disturbing the GIT ecosystem (56). These contentions are refuted by the present study and a recent report (60), which indicate that the GIT bacteria remain responsive to long-term feeding of prebiotics. Moreover, resistance to health challenges was increased by feeding inulin and oligofructose before the challenges, whereas switching rats to diets with NDO concurrent with exposure to carcinogens did not decrease the incidence of ACF, particularly in the proximal colon (51).

Finally, there is the question whether a dietary level of 100 g NDO/kg diet is relevant and that it causes a pharmacologic effect, not a physiologic response. Given a daily energy intake of 2000 kcal (8368 kJ), distributed as 40% from fat (9 kcal/g, 37.7 kJ) and 60% from carbohydrate and protein (each 4 kcal/g, 16.7 kJ), an individual would have to consume nearly 400 g of dry matter. The commonly recommended fiber intake of 30–40 g/d therefore represents ~7.5–10% of the dry matter consumed and is therefore comparable to the level of fiber in the diets fed to the mice. Although 40 g of NDO per day would exceed normal intake of fructans, this level of intake has been used in clinical trials (61) and does not exceed by orders of magnitude the normal intake of 1–10 g/d of fructans present in the Western diet (62). Although it remains contentious whether there is a dose-response relationship between dietary intake of fructans and increases in densities of LAB (61), comparable studies have yet to be reported for immune functions and other defense mechanisms.

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