J Microbiol Immunol Infect.
2009;42:451-456

Original Article

The effect of nonylphenol on the growth of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*

Jackson Tzu-Hsun Lin[1], Eddy-Essen Chang[2], Chih-Hsing Hung[3,4], Ay-Lin Huang[5], Tzu-Ching Wu[4], You-Hsuan Lee[5], Yi-Sheng Su[5], Chien-Jung Huang[6], Wan-Ting Hsu[7]

[1]Department of Pediatrics, Lin-Shin Hospital, Taichung; [2]Division of Nephrology, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung; [3]Department of Pediatrics, Faculty of Pediatrics, College of Medicine, Kaohsiung Medical University, Kaohsiung; [4]Department of Pediatrics, Kaohsiung Medical University Hospital, Kaohsiung; [5]Kaohsiung Municipal Hsin-chuang Senior High School, Kaohsiung; [6]Department of Meat and Milk Processing, Taiwan Livestock Research Institute, Hsinhua; and [7]Department of Clinical Pathology, Chung Shan Medical University Hospital, Taichung, Taiwan

Received: October 5, 2007 Revised: August 31, 2008 Accepted: November 19, 2008

Background and purpose: Nonylphenol (NP) is a well-known environmental hormone recognized as detrimental to the reproductive systems of aquatic animals and humans. The effect of NP on probiotics in the human gastrointestinal tract remains unclear. This study investigated the effect of NP on the growth of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*.

Methods: *L. acidophilus* and *B. bifidum* were grown in anaerobic cultures. Both strains were incubated with and without NP. The dose effects of NP on the growth of both probiotics were compared, and the effects of NP on the growth of *L. acidophilus* and *B. bifidum* in different concentrations were evaluated.

Results: NP 5 to 10 μg/mL inhibited the growth of *L. acidophilus* ($p < 0.05$), but was ineffective at 2.5 μg/mL ($p > 0.05$). NP significantly inhibited the growth of *B. bifidum* in a dose-dependent manner ($p < 0.05$). NP inhibited the growth of different concentrations of *L. acidophilus* (6.25×10^4 to 2.5×10^5 colony-forming units [CFU]/mL) and *B. bifidum* (1.25×10^9 to 5.0×10^9 CFU/mL) [$p < 0.05$].

Conclusions: Growth of *L. acidophilus* and *B. bifidum* was inhibited by NP. This finding suggests that NP may interfere with normal gastrointestinal microbiota. This may alter immunomodulation in the intestinal mucosa and may be correlated with an increase in the incidence of allergic diseases or other gastrointestinal disorders.

Key words: *Bifidobacterium*; Endocrine disrupters; Hypersensitivity; *Lactobacillus acidophilus*; Nonylphenol

Introduction

Nonylphenol (NP) is the most important metabolite of the group of non-ionic surfactants designated as nonylphenol polyethoxylates (NPnEO) [1], more popularly known as environmental hormones, environmental endocrine disrupting chemicals, or endocrine disruptors [2]. NP readily decomposes in the environment [3], so can influence human health via bioaccumulation in the diet. NP is structurally similar to 17β-estradiol, which can feminize male animals. The link of NP with infertility is unequivocal [4,5].

Probiotics are living microorganisms that benefit human health. Two widely studied examples are *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Both are Gram-positive anaerobic spore-forming lactic acid bacteria. *L. acidophilus* resides mainly in Peyer's patches and the small intestine. *B. bifidum* inhabits the colon, and is the predominant bacterial species in the intestines of breast-fed infants [6], where it presumably prevents colonization by potential pathogens. Consistent with this, the numbers of these

Corresponding author: Dr. Wan-Ting Hsu, Department of Clinical Pathology, Chung Shan Medical University Hospital, No.110, Sec. 1, Chien-Kuo North Rd., Taichung, Taiwan.
E-mail: shwantin@yahoo.com.tw

bacteria decline in the human body with age. Healthy infants tend to display higher levels of *B. bifidum* and more tenacious adhesion of fecal bifidobacteria than do infants with allergies, suggesting a correlation between allergic disease and intestinal bifidobacteria flora that have reduced adhesive abilities to the intestinal mucosa [7]. If so, this may be influential to human health, since disruption of the normal microbiota-mediated mechanism of immunomodulation in the mucosa leads to an increase in the incidence of allergic disease or other gastrointestinal (GI) disease [8-10].

In seeking to further the understanding of intestinal microbial flora, the ubiquity of NP in food has attracted attention [11]. A study conducted in Germany reported a daily intake of NP of 7.5 μg for an average German [11]. The daily intake of NP by adult Taiwanese people is estimated to be 35 μg [12], which is 4.5-fold that for Germans. After such long-term exposure to such an endocrine disruptor, the GI system will inevitably be affected. However, it is yet to be determined whether there is a close relationship between the normal flora of the human GI system and NP in the daily diet. This study investigated the effect of NP on the growth of *L. acidophilus and B. bifidum*, which are 2 important probiotics in humans.

Methods

This study was performed in vitro in anaerobic conditions that mimic the interaction between NP and *L. acidophilus and B. bifidum*. The effects of NP on the probiotics cultures were observed and quantified. NP was purchased from TCI (Tokyo, Japan). 69966 de Man, Rogosa, Sharpe (MRS) broth was purchased from Fluka GmbH (Buchs, Switzerland). *L. acidophilus* (Bioresources Collection and Research Center [BCRC] 10695, type strain B161) and *B. bifidum* (BCRC 14146, type strain B1) were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan).

Flora growth and counting

The suspension cultures of *L. acidophilus* or *B. bifidum* in the MRS broth were incubated at 37°C in anaerobic incubator for 24 h for optimal growth. Ten-fold serial dilutions into 10^{-1} to 10^{-10} bacterial cultures were made. Then, 10^{-3} to 10^{-9} diluted bacterial suspensions 0.1 mL were spread onto an MRS agar plate and incubated for 48 h for the colony counts. The effective range of colony counts were calculated as 25 to 250 colony-forming units (CFU)/per plate.

Interaction of probiotics and nonylphenol

As there are more than 300 different species of bacterium in the human gut [13], it is difficult to evaluate the effect of NP on the growth of each species of bacterium. The number of *Lactobacillus* spp. in the small intestine ranges from 10^5/g to 10^9/g, and that of *Bifidobacteria* spp. in the large intestine is 10^9/g to 10^{11}/g [14]. *L. acidophilus* 6.25 to 2.5×10^5 CFU/mL were used to represent the small intestinal probiotics and *B. bifidum* 1.25 to 5.0×10^9 CFU/mL represented the large intestinal probiotics.

The first group used *L. acidophilus* 1.25×10^5 CFU/mL with NP 2.50 μg/mL, 5.00 μg/mL, and 10.00 μg/mL. The second group used NP 5.00 μg/mL with *L. acidophilus* 6.25×10^4 CFU/mL, 1.25×10^5 CFU/mL, and 2.50×10^5 CFU/mL. The third group used *B. bifidum* with 2.50×10^9 CFU/mL with NP 2.50 μg/mL, 5.00 μg/mL, and 10.00 μg/mL. The fourth group used NP 5.00 μg/mL with *B. bifidum* 1.25×10^9 CFU/mL, 2.50×10^9 CFU/mL, and 5.00×10^9 CFU/mL. Each group contained NP 1 mL, original bacterial suspension 1 mL, and MRS broth 5 mL well mixed and incubated under an anaerobic incubator for 24 h, then 0.1 mL of broth was applied onto MRS agar plates. The plates were incubated under anaerobic conditions at 37°C for 48 h and the growth of *L. acidophilus* and *B. bifidum* were observed. The calculated bacterial concentrations were mean of colony counts × dilution 10 fold (10^n) × 10/volume of MRS plate. The effective range of colony counts was calculated as 25-250 CFU/per plate. Each experiment had a control group of probiotics only and a study group of probiotics with NP. All experiments were repeated 3 times by the same method.

Preparation of the original concentrations of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*

After the first incubation at 37°C for 24 h under anaerobic conditions, bacterial suspensions were diluted serially 10-fold with MRS broth and turned into 10^{-1} to 10^{-10} bacterial dilutions. The original bacterial suspensions were stoked immediately at 4°C after serial dilution. 10^{-3} to 10^{-9} diluted bacterial suspension 0.1 mL was applied to the MRS agar plates and incubated under anaerobic conditions at 37°C for 48 h and the colony counts were calculated. The effective range of colony counts were calculated as 25 to 250 CFU/per plate. The best-diluted concentration for *L. acidophilus* was 10^{-3}, the colony number was 175, and the calculated original bacterial concentration was 2.5×10^5 CFU/mL. The best diluted concentration for *B. bifidum* was 10^{-8}, the colony number

was 35, and the calculated bacterial concentration was 5×10^9 CFU/mL. For *L. acidophilus*, a diluted concentration of 10^{-3} was chosen, and the calculated bacterial concentration was 2.5×10^5 CFU/mL. This was diluted serially by 2-fold to form 1.25×10^5 CFU/mL and 6.25×10^4 CFU/mL. For *B. bifidum*, a diluted concentration of 10^{-8} was chosen and the calculated bacterial concentration was 5×10^9 CFU/mL. This was diluted serially by 2-fold to form 2.50×10^9 and 1.25×10^9 CFU/mL. *L. acidophilus* 6.25×10^4 to 2.50×10^5 CFU/mL were chosen to represent the small intestinal probiotics and *B. bifidum* 1.25×10^9 to 5.00×10^9 CFU/mL were chosen for the large intestinal probiotics.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Depending on the distribution of the data, the non-parametric Mann-Whitney *U* test was used to compare groups. There were significant differences in attrition among the 4 groups. A p value of <0.05 was considered to indicate statistical significance. All p values were 2-tailed. Statistical analyses were performed using the Statistical package for the Social Sciences for Windows (Version 10.0; SPSS, Inc., Chicago, IL, USA).

Results

Growth of *Lactobacillus acidophilus* with nonylphenol

Only higher or middle concentrations (5.0 μg/mL and 10.0 μg/mL), but not lower concentrations (2.5 μg/mL), of NP significantly inhibited the growth of *L. acidophilus*. Compared with the control group, lower concentrations of NP suppressed only 36.5% of *L. acidophilus* growth. Middle and higher doses suppressed 87.7% and 92.7% of *L. acidophilus* growth, respectively (Table 1).

Influence of nonylphenol on growth of *Lactobacillus acidophilus*

Compared with the control group, NP suppressed 48% of the lower concentration *L. acidophilus* growth,

Table 1. Growth of *Lactobacillus acidophilus* with different doses of nonylphenol.

Nonylphenol dose (μg/mL)	*Lactobacillus acidophilus* and nonylphenol (n = 6) CFU/mL; mean (SD)	*p*
0	86.40×10^6 (23.38×10^6)	
2.5	54.83×10^6 (20.86×10^6)	>0.05
5.0	10.67×10^6 (5.75×10^6)	0.004[a]
10.0	6.33×10^6 (3.01×10^6)	0.004[a]

[a]Significant difference ($p < 0.05$) when compared with the control group (nonylphenol, 0 μg/mL).

Table 2. The effect of nonylphenol 5 μg/mL on the growth of different concentrations of *Lactobacillus acidophilus*.

Lactobacillus acidophilus CFU/mL	*Lactobacillus acidophilus* and nonylphenol (n = 6) CFU/mL; mean (SD)	*Lactobacillus acidophilus* (n = 6) CFU/mL; mean (SD)	*p*
6.25×10^4	28.72×10^6 (7.90×10^6)	55.20×10^6 (15.91×10^6)	0.009
1.25×10^5	43.00×10^6 (9.07×10^6)	86.40×10^6 (23.38×10^6)	0.004[a]
2.50×10^5	55.00×10^6 (11.68×10^6)	127.08×10^6 (40.82×10^6)	0.002[a]

[a]Significant difference ($p < 0.05$) between the groups with and without nonylphenol.

Table 3. Growth of *Bifidobacterium bifidum* with different doses of nonylphenol.

Nonylphenol dose (μg/mL)	*Bifidobacterium bifidum* and nonylphenol (n = 6) CFU/mL; mean (SD)	*p*
0	152.83×10^6 (35.54×10^6)	
2.5	32.33×10^6 (11.76×10^6)	0.02[a]
5.0	18.33×10^6 (4.93×10^6)	0.02[a]
10.0	7.16×10^6 (4.21×10^6)	0.02[a]

[a]Significant difference ($p < 0.05$) when compared with the control group (nonylphenol, 0 μg/mL).

Table 4. The effect of nonylphenol 5 μg/mL on the growth of different concentrations of *Bifidobacterium bifidum*.

Bifidobacterium bifidum CFU/mL	*Bifidobacterium bifidum* and nonylphenol (n = 6) CFU/mL; mean (SD)	*Bifidobacterium* bifidum (n = 6) CFU/mL; mean (SD)	*p*
1.25×10^9	40.83×10^6 (5.78×10^6)	150.0×10^6 (61.23×10^6)	0.01
2.50×10^9	55.00×10^6 (7.62×10^6)	152.83×10^6 (35.54×10^6)	0.02[a]
5.00×10^9	71.83×10^6 (15.90×10^6)	187.17×10^6 (34.93×10^6)	0.02[a]

[a]Significant difference ($p < 0.05$) between the groups with and without nonylphenol.

50.2% of the middle concentration *L. acidophilus* growth, and 56.7% of the high concentration *L. acidophilus* growth. NP 5 μg/mL inhibited *L. acidophilus* growth at different concentrations, as shown in Table 2.

Growth of *Bifidobacterium bifidum* with nonylphenol

Compared with the control group, lower concentrations (2.5 μg/mL) of NP suppressed 78.8% of *B. bifidum* growth. Middle (5 μg/mL) and higher (10 μg/mL) doses of NP suppressed 88.0% and 95.3% of *B. bifidum* growth, respectively (Table 3).

Influence of nonylphenol on growth of *Bifidobacterium bifidum*

Compared with the control group, NP 5 μg/mL suppressed 72.8% of the lower concentration *B. bifidum* growth, 64.0% of the middle concentration *B. bifidum* growth, and 61.6% of the high concentration *B. bifidum* growth. NP 5 μg/mL significantly inhibited *B. bifidum* growth at different concentrations, as shown in Table 4.

Discussion

This study investigated the growth effect of *L. acidophilus* and *B. bifidum* with different dosages of NP, and the growth effect of different concentrations of both probiotics with and without the presence of NP. When NP was first discovered in 1984 [1], it became a global concern. Many studies of NP have focused on the reproductive or endocrine systems of animals and humans [2-5]. In recent years, several studies have investigated the effect of NP on the immune system [15-17]. In this study, NP was shown to inhibit the growth of *L. acidophilus* and *B. bifidum*, 2 important probiotics in the gut. This action of NP may interfere with the balance of intestinal microflora.

NP can bioaccumulate via contact, inhalation, and diet. The compound is commonly prevalent in food. Once ingested, elimination of NP from the intestinal tract may be slow, due to the impaired function of methylenedioxyphenyl-glucuronosyltransferase [18]. This study demonstrated that NP is capable of hindering the growth of *L. acidophilus* and *B. bifidum* in vitro. If occurring in vivo, this would disrupt the intestinal microflora. A quantification method was used to calculate the bacterial colony in this study because of the accuracy. If the inhibition effect was easily induced in vitro, then it is reasonable to expect that the inhibition effect could also be induced in vivo.

Although many studies have investigated NP [2-5,11,12,15-17], there are few data for the effects of NP effects on the GI system. The pharmacokinetics of NP in humans is still unclear, but the absorption, bioavailability, metabolism, and excretion of NP in a rat model has been investigated [19]. Green et al found that, after feeding rats with NP, the radioactivity concentrations of 14C-NP in the feces was 600 to 1400 times more than in plasma [19]. As the NP plasma levels of humans ranges from non-detectable to 6.77 ng/mL [20], the concentrations of NP used in this study to simulated NP in the intestinal level ranged from 2.5 μg/mL to 10 μg/mL.

In this study, there was no significant difference between the control group and NP 2.5 μg/mL on the growth of *L. acidophilus*. NP inhibited growth of different concentrations of *L. acidophilus*. NP also suppressed *B. bifidum* growth at low doses and in a dose-dependent manner. NP significantly inhibited *B. bifidum* growth at different concentrations.

As there are more than 300 different species of bacterium in the human gut, it is difficult to evaluate the effect of NP on the growth of each species. *L. acidophilus* 6.25×10^4 to 2.50×10^5 CFU/mL was used to represent the small intestinal probiotics and *B. bifidum* 1.25×10^9 to 5.00×10^9 CFU/mL was used to represent the large intestinal probiotics. Probiotics are cultures of potentially beneficial bacteria of the healthy gut microflora. Various strains of *Lactobacilli* and *Bifidobacteria* have been reported to bestow an array of health-promoting activities after either parenteral or oral administration, including improved

resistance to intestinal infections, antimutagenic activity, control of serum cholesterol, alleviation of lactose intolerance, and positive effects on diarrhea, allergies, and autoimmunity [21].

The connection between NP and diet appears to be valid. Maternal transfer of NP may occur [22,23] and may be linked to the induction of estrogen expression [22]. There is no reason to think that the influence of NP does not extend to the immune system. The results of this study support this view. The fact that there is no data concerning the safe daily intake of NP by humans is disturbing. While some countries in Europe have proactively decreased or stopped the use of nonionic surfactants [24], the use of nonionic surfactants in industrial and household products in Taiwan is common. Many studies have focused on the reproductive system, but the digestive and immune systems may also be involved in pollution by NP in the diet and via inhalation, contact, breast-feeding, or maternal-fetal transfer. The problem will become more serious if the use of detergents and cleansers are not well controlled.

NP significantly inhibits the growth of both *L. acidophilus* and *B. bifidum* in vitro. Therefore, NP may affect the growth of some normal flora and change the balance of microbiota in the human gut, thereby inducing intestinal disease. NP-contaminated food may affect not only the human endocrine, reproductive, and immune systems, but also the digestive system.

Acknowledgment

Technical support from Ms. Min-Ru Tasi is acknowledged.

References

1. Giger W, Brunner PH, Schaffner C. 4-Nonylphenol in sewage sludge: accumulation of toxic metabolites from nonionic surfactants. Science. 1984;225:623-5.
2. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology. 1994;135:175-82.
3. Correa-Reyes G, Viana MT, Marquez-Rocha FJ, Licea AF, Ponce E, Vazquez-Duhalt R. Nonylphenol algal bioaccumulation and its effect through the trophic chain. Chemosphere. 2007;68:662-70.
4. Popek W, Dietrich G, Glogowski J, Demska-Zakes K, Drag-Kozak E, Sionkowski J, et al. Influence of heavy metals and 4-nonylphenol on reproductive function in fish. Reprod Biol. 2006;6:175-88.
5. Fraser LR, Beyret E, Milligan SR, Adeoya-Osiguwa SA. Effects of estrogenic xenobiotics on human and mouse spermatozoa. Hum Reprod. 2006;21:1184-93.
6. Beerens H, Romond C, Neut C. Influence of breast-feeding on the bifid flora of the newborn intestine. Am J Clin Nutr. 1980;33:2434-9.
7. He F, Ouwehand AC, Isolauri E, Hashimoto H, Benno Y, Salminen S. Comparison of mucosal adhesion and species identification of bifidobacteria isolated from healthy and allergic infants. FEMS Immunol Med Microbiol. 2001;30:43-7.
8. Rautava S, Kalliomaki M, Isolauri E. New therapeutic strategy for combating the increasing burden of allergic disease: Probiotics-A Nutrition, Allergy, Mucosal Immunology and Intestinal Microbiota (NAMI) Research Group report. J Allergy Clin Immunol. 2005;116:31-7.
9. Penders J, Thijs C, van den Brandt PA, Kummeling I, Snijders B, Stelma F, et al. Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. Gut. 2007;56:661-7.
10. Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, et al. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. J Allergy Clin Immunol. 2007;119:192-8.
11. Guenther K, Heinke V, Thiele B, Kleist E, Prast H, Raecker T. Endocrine disrupting nonylphenols are ubiquitous in food. Environ Sci Technol. 2002;36:1676-80.
12. Lu YY, Chen ML, Sung FC, Paulus Shyi-Gang W, Mao IF. Daily intake of 4-nonylphenol in Taiwanese. Environ Int. 2007;33:903-10.
13. Simon GL, Gorbach SL. Intestinal flora in health and disease. Gastroenterology. 1984;86:174-93.
14. Mitsuoka T. Intestinal flora and aging. Nutr Rev. 1992;50:438-46.
15. Estevez MC, Kreuzer M, Sanchez-Baeza F, Marco MP. Analysis of nonylphenol: advances and improvements in the immunochemical determination using antibodies raised against the technical mixture and hydrophilic immunoreagents. Environ Sci Technol. 2006;40:559-68.
16. Lee MH, Chung SW, Kang BY, Park J, Lee CH, Hwang SY, et al. Enhanced interleukin-4 production in CD4+ T cells and elevated immunoglobulin E levels in antigen-primed mice by bisphenol A and nonylphenol, endocrine disruptors: involvement of nuclear factor-AT and Ca^{2+}. Immunology. 2003;109:76-86.
17. Yao G, Yang L, Hu Y, Liang J, Liang J, Hou Y. Nonylphenol-induced thymocyte apoptosis involved caspase-3 activation and mitochondrial depolarization. Mol Immunol. 2006;43:915-26.
18. Daidoji T, Ozawa M, Sakamoto H, Sako T, Inoue H,

Kurihara R, et al. Slow elimination of nonylphenol from rat intestine. Drug Metab Dispos. 2006;34:184-90.
19. Green T, Swain C, Van Miller JP, Joiner RL. Absorption, bioavailability, and metabolism of para-nonylphenol in the rat. Regul Toxicol Pharmacol. 2003;38:43-51.
20. Lu JP, Zheng LX, Cai DP. Study on the level of environmental endocrine disruptors in serum of precocious puberty patients. Zhonghua Yu Fang Yi Xue Za Zhi. 2006;40:88-92. [Article in Chinese.]
21. Naidu AS, Bidlack WR, Clemens RA. Probiotic spectra of lactic acid bacteria (LAB). Crit Rev Food Sci Nutr. 1999; 39:13-26.
22. Hong EJ, Choi KC, Jung YW, Leung PC, Jeung EB. Transfer of maternally injected endocrine disruptors through breast milk during lactation induces neonatal Calbindin-D9k in the rat model. Reprod Toxicol. 2004;18:661-8.
23. Sakurai K, Mori C. Fetal exposure to endocrine disruptors. Nippon Rinsho. 2000;58:2508-13. [Article in Japanese.]
24. Renner R. European bans on surfactant trigger transatlantic debate. Environ Sci Technol. 1997;31:316-20.