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

Jackson Tzu-Hsun Lin¹, Eddy-Essen Chang², Chih-Hsing Hung¹,⁴, Ay-Lin Huang⁵, Tzu-Ching Wu¹, You-Hsuan Lee⁵, Yi-Sheng Su⁵, Chien-Jung Huang⁶, Wan-Ting Hsu⁷

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

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**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 × 10⁴ to 2.5 × 10⁵ colony-forming units [CFU/mL]) and *B. bifidum* (1.25 × 10⁹ to 5.0 × 10⁹ 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
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. Tenfold 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 bacteria 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 10^7^ g to 10^10^ 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 × 10^6^ CFU/mL, 2.50 × 10^6^ CFU/mL, and 5.00 × 10^6^ 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 (10^9^ × 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^-3^ 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^-4^, 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 $5 \times 10^6$ CFU/mL. For L. acidophilus, a diluted concentration of $10^{-3}$ was chosen, and the calculated bacterial concentration was $2.5 \times 10^5$ CFU/mL. This was diluted serially by 2-fold to form $1.25 \times 10^5$ CFU/mL and $6.25 \times 10^4$ CFU/mL. For B. bifidum, a diluted concentration of $10^{-8}$ was chosen and the calculated bacterial concentration was $5 \times 10^9$ CFU/mL. This was diluted serially by 2-fold to form $2.50 \times 10^9$ and $1.25 \times 10^9$ CFU/mL. L. acidophilus $6.25 \times 10^4$ to $2.50 \times 10^5$ CFU/mL were chosen to represent the small intestinal probiotics and B. bifidum $1.25 \times 10^9$ to $5.00 \times 10^9$ CFU/mL were chosen for the large intestinal probiotics.

Statistical analysis
The results were expressed as mean $\pm$ 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 ($5.0 \mu g/mL$ and $10.0 \mu g/mL$), but not lower concentrations ($2.5 \mu g/mL$), 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 2. The effect of nonylphenol 5 $\mu g/mL$ on the growth of different concentrations of Lactobacillus acidophilus.

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

<table>
<thead>
<tr>
<th>Nonylphenol dose ($\mu g/mL$)</th>
<th>Lactobacillus acidophilus and nonylphenol (n = 6)</th>
<th>CFU/mL; mean (SD)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>86.40 $\times 10^6$ (23.38 $\times 10^6$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>54.83 $\times 10^6$ (20.86 $\times 10^6$)</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>10.67 $\times 10^6$ (5.75 $\times 10^6$)</td>
<td>0.004*</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>6.33 $\times 10^6$ (3.01 $\times 10^6$)</td>
<td>0.004*</td>
<td></td>
</tr>
</tbody>
</table>

*Significant difference ($p < 0.05$) when compared with the control group (nonylphenol, 0 $\mu g/mL$).

Table 2. The effect of nonylphenol 5 $\mu g/mL$ on the growth of different concentrations of Lactobacillus acidophilus.

<table>
<thead>
<tr>
<th>Lactobacillus acidophilus CFU/mL</th>
<th>Lactobacillus acidophilus and nonylphenol (n = 6) CFU/mL; mean (SD)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25 $\times 10^4$</td>
<td>28.72 $\times 10^6$ (7.90 $\times 10^6$)</td>
<td>0.009</td>
</tr>
<tr>
<td>1.25 $\times 10^5$</td>
<td>43.00 $\times 10^6$ (9.07 $\times 10^6$)</td>
<td>0.004*</td>
</tr>
<tr>
<td>2.50 $\times 10^5$</td>
<td>55.00 $\times 10^6$ (11.68 $\times 10^6$)</td>
<td>0.002*</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Nonylphenol dose ($\mu g/mL$)</th>
<th>Bifidobacterium bifidum and nonylphenol (n = 6) CFU/mL; mean (SD)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>152.83 $\times 10^8$ (35.54 $\times 10^8$)</td>
<td>0.02*</td>
</tr>
<tr>
<td>2.5</td>
<td>32.33 $\times 10^8$ (11.76 $\times 10^8$)</td>
<td>0.02*</td>
</tr>
<tr>
<td>5.0</td>
<td>18.33 $\times 10^8$ (4.93 $\times 10^8$)</td>
<td>0.02*</td>
</tr>
<tr>
<td>10.0</td>
<td>7.16 $\times 10^8$ (4.21 $\times 10^8$)</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

*Significant difference ($p < 0.05$) when compared with the control group (nonylphenol, 0 $\mu g/mL$).
Nonylphenol and probiotic growth

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

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>Growth of <em>Bifidobacterium bifidum</em> and nonylphenol (n = 6) mean (SD)</th>
<th>Growth of <em>Bifidobacterium bifidum</em> (n = 6) mean (SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25 × 10^9</td>
<td>40.83 × 10^6 (5.78 × 10^6)</td>
<td>150.0 × 10^6 (61.23 × 10^6)</td>
<td>0.01</td>
</tr>
<tr>
<td>2.50 × 10^9</td>
<td>55.00 × 10^6 (7.62 × 10^6)</td>
<td>152.83 × 10^6 (35.54 × 10^6)</td>
<td>0.02a</td>
</tr>
<tr>
<td>5.00 × 10^9</td>
<td>71.83 × 10^6 (15.90 × 10^6)</td>
<td>187.17 × 10^6 (34.93 × 10^6)</td>
<td>0.02a</td>
</tr>
</tbody>
</table>

*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 bacteria in the human gut, it is difficult to evaluate the effect of NP on the growth of each species. *L. acidophilus* 6.25 × 10^7 to 2.50 × 10^8 CFU/mL was used to represent the small intestinal probiotics and *B. bifidum* 1.25 × 10^7 to 5.00 × 10^8 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**

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**References**

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