

## *Bacillus clausii* Probiotic Strains Antimicrobial and Immunomodulatory Activities

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**Abstract:** The clinical benefits observed with probiotic use are mainly attributed to the antimicrobial substances produced by probiotic strains and to their immunomodulatory effects. Currently, the best-documented probiotic bacteria used in human therapy are lactic acid bacteria. In contrast, studies aiming to characterize the mechanisms responsible for the probiotic beneficial effects of *Bacillus* are rare. The current work seeks to contribute to such characterization by evaluating the antimicrobial and immunomodulatory activities of probiotic *B. clausii* strains. *B. clausii* strains release antimicrobial substances in the medium. Moreover, the release of these antimicrobial substances was observed during stationary growth phase and coincided with sporulation. These substances were active against Gram-positive bacteria, in particular against *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridium difficile*. The antimicrobial activity was resistant to subtilisin, proteinase K, and chymotrypsin treatment, whereas it was sensitive to pronase treatment. The evaluation of the immunomodulatory properties of probiotic *B. clausii* strains was performed in vitro on Swiss and C57 Bl/6j murine cells. The authors demonstrate that these strains, in their vegetative forms, are able to induce NOS II synthetase activity, IFN- $\gamma$  production, and CD4<sup>+</sup> T-cell proliferation.

**Key Words:** *Bacillus clausii*, antimicrobial substance, immunostimulation

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The use of probiotics to enhance human health has been proposed for many years.<sup>1</sup> Recently, the term *probiotics* was redefined as “live microorganisms administered in an adequate amount which confer a health benefit on the host.”<sup>2</sup> A significant number of studies have demonstrated the therapeutic efficacy of probiotics when applied to the treatment of several gastrointestinal microbial disorders, including acute diarrhea, antibiotic-associated diarrhea, and traveler’s diarrhea.<sup>3–5</sup> Be-

side their therapeutic use as pharmaceuticals, probiotics have been extensively included during the past 2 decades in various types of food products (in particular, those based on fermented milk).

So far, the best-documented probiotics are lactic acid bacteria. In contrast, the mechanisms responsible for the beneficial effects of other probiotics, especially the *Bacillus* species, have remained relatively unexplored until fairly recently. *Bacillus* strains for animal nutritional use and for human use (pharmaceutical preparations) include *B. subtilis*, *B. licheniformis*, *B. coagulans*, *B. toyoi* (*cereus*), *B. natto* (*subtilis*), *B. clausii*, *B. polyfermentans*, and *B. cereus*.<sup>6–9</sup>

Many aspects, including safety and functional characteristics, need to be considered during the selection of microorganisms for probiotic design. Functional characteristics include viability and persistence in the gastrointestinal tract, antagonistic properties against bacterial infection, and immunomodulation. The antimicrobial activity of probiotic bacteria was suggested to be one of the probiotic pathogen-inhibiting mechanisms. Moreover, several authors have previously reported that antimicrobial activity of probiotic bacteria in vitro and in vivo is mainly attributed to their capacity to produce antimicrobial substances. Recent reports suggest that the antimicrobial activity of *Lactobacillus* probiotics involves the production of secreted compounds, such as organic acid, hydrogen peroxide, as well as various antibiotics and/or bacteriocins.<sup>10–13</sup> Our recent study demonstrated that the antibacterial activity of one *B. subtilis* probiotic strain (Biosporin) is the result of the production of 2 distinct antibiotics.<sup>8</sup>

In addition, several studies have demonstrated that orally administered probiotic bacteria can stimulate the cellular and humoral immune systems, resulting in health improvement during intestinal infections.<sup>14,15</sup> *Bacillus* probiotics have been reported to induce cellular and humoral immune responses in vivo.<sup>16–18</sup> Recent studies have shown that orally ingested *B. subtilis* spores are immunogenic, can disseminate to the Payer’s patches and mesenteric lymph nodes, and probably affect cytokine production.<sup>19</sup> Moreover, given their immunomodulatory effects, these bacteria were proposed for use as an oral vaccine delivery vehicle.<sup>19,20</sup> Despite the considerable recent advances in the characterization of *Bacillus* probiotics, the molecular and cellular mechanisms by which

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these bacteria exert their immune modulatory action are still unknown.

The *Bacillus* probiotic Enterogermina, which includes 4 strains of *Bacillus*, recently reclassified from *subtilis* to *clausii* and shown to display a low level of intraspecific diversity,<sup>21</sup> has been reported to exert beneficial clinical effects, notably in the treatment of diarrhea and in the prevention of infectious diseases.<sup>3,16</sup> However, the precise mechanisms responsible for these effects remain unclear. The current study was designed to analyze the antimicrobial activity of the *B. clausii* strains and to explore their immunomodulatory action through their ability to activate murine antigen presenting cells and T-cell subsets in vitro.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Media

The 4 *B. clausii* probiotic strains O/C, N/R, SIN, and T (Enterogermina); *B. subtilis* 3 (Biosporine), *B. licheniformis* 31 (Biosporine), *B. cereus* IP 5832 (Bactisubtil), *B. cereus* NT (Biosubtyl), and *B. cereus* DM-423 (Cereobiogen); and the reference strain *B. subtilis* 168 were used in the current study. The strains used as test cultures (in the assays of antimicrobial activity) are listed in Table 1. *Bacillus* strains were cultivated in Mueller Hinton (MH) broth (Difco Laboratory, Detroit, MI)

**TABLE 1.** Inhibitory Activity Produced by *B. clausii* OC Supernatant for Different Pathogenic and Nonpathogenic Intestinal Bacteria and Other Microorganisms

Strain Tested	Activity*
<i>S. aureus</i> CIP† 350 53 156	++
<i>Enterococcus faecium</i> LMBA‡ 27323	+
<i>E. faecium</i> LMBA 27323	+
<i>Micrococcus</i> sp LMBA 26	++
<i>Lactococcus lactis</i> ATCC§ 11454	+
<i>L. lactis</i> LMBA 374	+
<i>Clostridium difficile</i> 514¶	++
<i>Escherichia coli</i> LMBA 20684	-
<i>Salmonella enterica</i> serovar; <i>S. typhimurium</i> ATCC 29629	-
<i>S. flexneri</i> LMBA 12225	-
<i>Vibrio cholerae</i> NCTC** 8021	-
<i>V. parahaemolyticus</i> ATCC 17802	-
<i>Pseudomonas fluorescens</i> LMBA BE1	-
<i>Fusarium oxysporum</i> ox52††	-

\*Antimicrobial activity determined by agar diffusion test.

†Collection de l'Institut Pasteur.

‡LMBA, laboratoire de Microbiologie et Biochimie Appliquée, ENTIA de Bordeaux, France.

§ATCC, American Type Culture Collection.

¶Hopital Pellegrin, Bordeaux, France.

\*\*National Collection of Type Cultures, England.

††INRA de Bordeaux.

or on MH agar for 24 to 72 hours at 30°C. Lactic acid bacteria were grown in MRS (de Man, Rogosa, and Sharpe) broth (Difco Laboratories) for 24 hours at 37°C. The other bacteria listed in Table 1 were grown in brain heart infusion (BHI) media (Difco). BHI medium supplemented with 2% NaCl was used for the *vibrio* species growth. *Clostridium difficile* was grown in *Brucella* agar (Anaerobe Systems, Morgan Hill, CA) supplemented with 5% defibrinated sheep blood. Finally, the Malta agar was used for fungi cultures. The sporulation percentage determination of *B. clausii* strains grown in different conditions was determined after Gram staining and microscopic observation.

### Determination of Antimicrobial Activity

A colony overlay assay was used for the detection of probiotic antimicrobial activity.<sup>22</sup> The presence of antagonistic activity of the *Bacillus* probiotic strains was determined as an inhibition of test culture growth around the *Bacillus* spot. An agar well diffusion method was used for analysis of *Bacillus* culture supernatant antimicrobial activity, as described previously.<sup>23</sup> The titer of antimicrobial activity (activity units [AU] per milliliter) was defined as the supernatant with highest dilution showing inhibition of *S. aureus* CIP 350 53 156.

To characterize the antimicrobials produced by probiotic bacteria, we analyzed the thermostability and enzymatic treatment resistance of the antimicrobials present in culture supernatant of the probiotic strains. Analyses were performed as described previously.<sup>24</sup> Enzymes (subtilisin, proteinase K, chymotrypsin, pronase, lipase, α-amylase, and lysozyme) were used at final concentrations of 1 mg/ml. The anti-*S. aureus* activity was determined before and after each treatment using an agar well diffusion method.

### Evaluation of Immunomodulatory Activity

The murine peritoneal and spleen cells were isolated from female Swiss albino and C57BL/6j mice (6–12 weeks old; Iffa Credo, St. Germain sur l'Abresle, France) as described previously.<sup>25</sup> Briefly, cells were washed twice and resuspended in RPMI 1640 (Sigma) medium with supplements containing 10% fetal calf serum (Sigma), 2 mM L-glutamate (Sigma), 2 mM sodium pyruvate (Sigma), 20 mM HEPES (Sigma), and 25 µg/mL gentamicin (RPMI-FCS). Murine CD4<sup>+</sup> T cells were purified from the spleen cells by an anti-CD4<sup>+</sup> magnetic bead selection method (Meltanyi Biotec, CA).

A total of 5 × 10<sup>5</sup> CFU of probiotic strains were added to 5 × 10<sup>5</sup> peritoneal or spleen cell cultures. Cocultures were realized in 96 well plates. The plates were incubated 72 hours.

The concentration of nitrite (NO<sub>2</sub><sup>-</sup>), a stable oxidized derivative of NO, was determined spectrophotometrically at 540 nm with Griess reagents as previously described.<sup>25</sup>

IFN-γ production was measured in supernatants of murine spleen cells exposed to *Bacillus* probiotic bacteria using

ELISA (PharMingen, CA) according to the manufacturer's instructions.

T-cell proliferation assay was used to determine whether *Bacillus* probiotic bacteria could activate T-cell proliferation in the presence of antigen presenting cells (APC). Briefly, the assay was performed in 96 well plates, in which  $5 \times 10^5$  irradiated (3300 rad) naive spleen cells were used (as APC) in coculture with  $2 \times 10^5$  purified CD4<sup>+</sup> T cells in the presence or absence of *Bacillus* probiotics ( $5 \times 10^5$  CFU/well). The plates were incubated for 4 days at 37°C, 5% CO<sub>2</sub>. Eighteen hours before the end of coculture, the cells were pulsed with [<sup>3</sup>H]-thymidine (1 μCi; ICN Pharmaceuticals Inc., CA). Finally, [<sup>3</sup>H]-thymidine incorporation was assessed using a liquid scintillation counter (Beckman Instrument Inc.).

## RESULTS

### Antimicrobial Activity

The antagonistic activity of *B. clausii* probiotic strains (O/C, N/R, SIN, and T) was analyzed using colony overlay assay. In these experiments, *S. aureus* and *Salmonella* strains were used as a test culture. All tested *B. clausii* strains exhibited anti-staphylococcal activity, but not anti-*Salmonella* activity in vitro. The cell-free supernatant of the *B. clausii* O/C strain was used for further characterization of the antimicrobials produced.

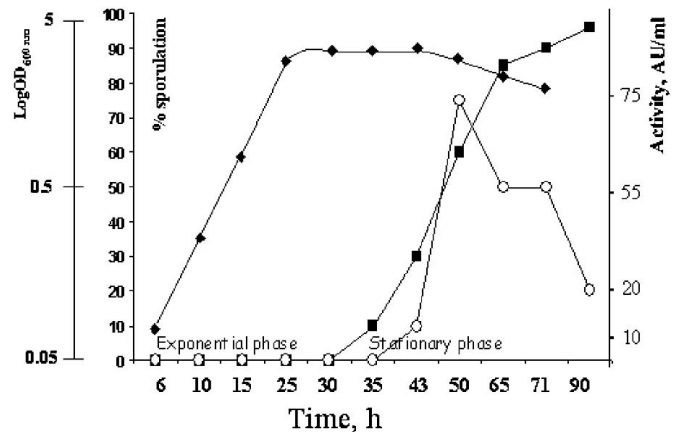
The antimicrobial compounds present in the cell-free supernatant of *B. clausii* O/C displayed a relatively narrow activity. Among the tested bacteria, only Gram-positive species, including *C. difficile*, were inhibited. No inhibitory effect was observed against Gram-negative bacteria and fungi (Table 1).

The time course study of antimicrobial production was realized when O/C strain was grown in MH broth. The anti-staphylococcal activity of the cell-free supernatant was followed during different growth phases (Fig. 1). Significant production of compounds carrying anti-staphylococcal activity started in the middle of the stationary growth phase (35–43 hours of culture growth) and coincided with sporulation. The maximum level of antimicrobial production (74 UA/mL) was achieved when the sporulation rate was 60%.

The antimicrobial compounds present in the cell-free supernatant were relatively thermostable, because activity remained after 30 minutes of incubation at 85°C. Moreover, the cell-free supernatant kept 60% of anti-staphylococcal activity after 30 minutes of heating at 95°C. Treatment of the cell-free supernatant with subtilisin, proteinase K, chymotrypsin, lipase, α-amylase, or lysozyme did not affect its antimicrobial activity. However, the supernatant was inactivated by pronase treatment.

### Immunomodulatory Activity

To characterize the immunomodulatory activity of *B. clausii* probiotic strains, these bacteria were screened for



**FIGURE 1.** Growth and production of an antimicrobial substance by probiotic strain *B. clausii* O/C. During growth, optical density (OD) at 600 nm of culture (◆), percentage of sporulation (■), and anti-*S. aureus* activity in activity units per milliliter (○) was measured.

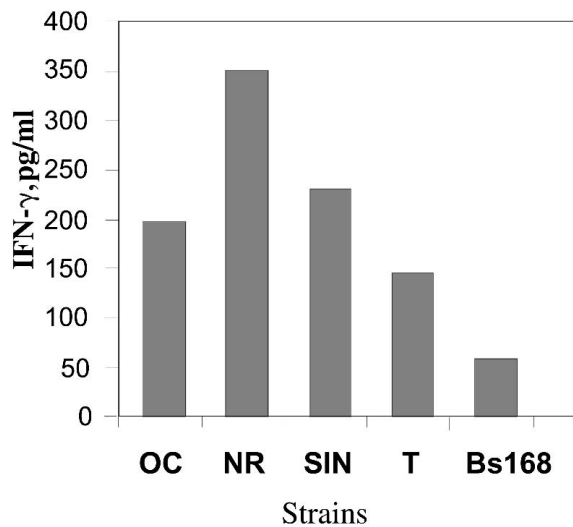
their ability to stimulate nitrite production in Swiss murine peritoneal cells. Nitrite concentrations were measured in 72-hour coculture supernatants. The vegetative cells of the *B. clausii* strains induced significant levels of nitrite production (approximately 100 μM). Moreover, these levels of stimulation were comparable with those obtained when peritoneal cells were cocultured with *B. subtilis* 3 probiotic strain. Other tested vegetative cells of *Bacillus* probiotics (*B. licheniformis* 31, *B. cereus* IP 5832, *B. cereus* NT, and *B. cereus* DM-423) and the reference strain *B. subtilis* 168 had a lower stimulatory effect (nitrite concentration range, 35–75 μM). This increase in nitrite production was completely abolished when L-N<sup>6</sup>-(imino-ethyl)-lysine-dihydrochloride (L-NIL) was added, suggesting that the stimulatory effect occurred through NOS II induction.

NOS II activity in murine APC can be induced by certain cytokines, including INF-γ. We thus analyzed the effect of the four *B. clausii* probiotic strains on IFN-γ production by murine C57 BL/6j spleen cells using ELISA. All 4 strains showed a strong stimulatory effect on IFN-γ production, which was significantly higher than the effect observed with the *B. subtilis* 168 reference strain (Fig. 2).

Finally, we analyzed the ability of *B. clausii* strains to activate CD4<sup>+</sup> T-cell proliferation in the presence of APC. The CD4<sup>+</sup> T cells were purified from C57Bl/6j murine spleen cells. The purity of these cells was 96 to 98% (flow cytometry analysis, data not shown). All *B. clausii* strains induced a significant T-cell proliferative response (Table 2). The probiotic strain lymphoproliferation stimulatory effect was comparable with that obtained with concanavalin A.

## DISCUSSION

The concept of orally taken nonpathogenic microorganisms for the improvement of one's health is not new. However,



**FIGURE 2.** Stimulation of IFN- $\gamma$  production by murine spleen cells induced with *B. clausii* probiotic strains. Spleen cells were isolated from C57BL/6j mice and cocultured with probiotic strain *B. clausii* OC (O/C), *B. clausii* NR (N/R), *B. clausii* SIN (SIN), *B. clausii* T (T), or reference strain *B. subtilis* 168 (Bs168). Analysis of IFN- $\gamma$  production by murine spleen cells was realized 72 hours later using ELISA.

this idea has not received serious attention until recently. The lack of medical acceptance has been the result of a previous lack of scientifically reported evidence of the beneficial effects of probiotics. During the last decade, clinical studies have lent support to the use of selected probiotic agents for the prevention and treatment of gastrointestinal disorders.<sup>26-27</sup> (Hart, 2003). However, little is known about the mechanisms responsible for the clinically observed effects. Recent studies indicate that probiotics probably work by multiple mechanisms.<sup>8,14,15</sup> Furthermore, each agent may have a unique action.

**TABLE 2.** *B. clausii* Probiotic Strains Stimulate Proliferation of Murine CD4<sup>+</sup> T Cells

Strain/Immunostimulator	Stimulation of Murine CD4 <sup>+</sup> T Cell* Proliferation, Index of Stimulation
<i>B. clausii</i> OC	2.5
<i>B. clausii</i> NR	3.8
<i>B. clausii</i> SIN	2.7
<i>B. clausii</i> T	2.9
<i>B. subtilis</i> 168	1.9
Con A, 2 $\mu$ g/mL†	3.4

\*A total of  $5 \times 10^5$  CFU bacteria was used for stimulation of  $2 \times 10^5$  CD4<sup>+</sup> T cells in the presence of irradiated (3300 rad) naive murine spleen cells ( $5 \times 10^5$  cells). Spleen and CD4<sup>+</sup> T cells were isolated from C57Bl/6j mice.

†Concanavalin A was used as a positive control for CD4<sup>+</sup> T-cell stimulation.

Bacteria of *Bacillus* genus are known as producers of a large number of bacteriocins and antibiotics. However, little information is available regarding the antimicrobials produced by *Bacillus* probiotic strains. The commercial probiotic *B. polyfermentans* SCD, which has been successfully used for the treatment of long-term intestinal disorders, was described to produce polyfermenticin (proteinase K-sensitive and heat labile bacteriocin).<sup>7</sup> We also reported that probiotic strain *B. subtilis* 3 (Biosporin) produced at least two antimicrobial substances, one of which was identified as Amicoumacin A.<sup>8</sup> Moreover, our recent data demonstrate that this strain also produces four other antibiotics of a lipopeptide nature (personal communication). In the current work we demonstrated that all four *B. clausii* strains, which are included in the probiotic Enterogermina, exhibited an antimicrobial activity in vitro. Moreover, we determined that *B. clausii* O/C strain produced at least one antimicrobial substance secreted in the media, which displayed an activity against the Gram-positive bacteria. This substance was characterized as a bacteriocin-like substance, because its antimicrobial activity was sensitive to pronase treatment. However, the time course analysis of the substance production demonstrated that this antimicrobial had secondary-metabolite production kinetics, in contrast to bacteriocins that are considered to be primary metabolites. Therefore we do not exclude that the produced substance could be an antibiotic of polypeptide or lipopeptide nature, because such antibiotics were described to be frequently produced by many *Bacillus* species.<sup>7,8,23,26</sup>

The use of probiotics for the treatment of primary and recurrent *C. difficile* diarrhea was recently proposed.<sup>26,28</sup> Interestingly, the antimicrobial substance produced by *B. clausii* O/C strain was active against the *C. difficile* strains tested. This in vitro-observed anti-*C. difficile* effect opens perspectives for the therapeutic benefits of Enterogermina use in the treatment of *C. difficile*-associated diarrhea.

The precise mechanisms by which probiotics improve host defenses and mediate protection are not fully known. There is evidence to suggest that probiotics might also contribute to host health by stimulating both specific and nonspecific host immune responses. Numerous studies concerning the immunomodulatory properties of lactic acid bacteria strains have been recently published.<sup>14,15</sup> As for *Bacillus* probiotics, little information is available. It has been suggested that the nitrite formation resulting from NOS II activity might be involved in the T<sub>H</sub>1/T<sub>H</sub>2 balance.<sup>29</sup> In our study, all *Bacillus* probiotics tested induced NOS II expression and consequently nitrite production in macrophages, after coculturing the bacteria with murine peritoneal cells. This NOS II induction effect was indirect, because no nitrite production was observed in the coculture of the bacteria with purified macrophages or J774.2 cell line (data not shown).

The immunomodulating activity of lactic acid bacteria may be attributed, in part, to the involvement of a cytokine



network, which plays a pivotal role in coordinating immune function.<sup>30,31</sup> Muscettola et al,<sup>17</sup> demonstrated that administration of the probiotic Enterogermina, containing 4 *B. clausii* strains, to mice increased ex vivo IFN production. In our study we demonstrated that in vitro each of these 4 *B. clausii* probiotic strains stimulated the production of IFN- $\gamma$  by murine spleen cells. Moreover, all these strains induced the proliferation of murine CD4<sup>+</sup> T cells in the presence of irradiated APC. The immunomodulatory capacity of these strains could be the result of the expression of some extracellular and/or cell wall-associated compounds involved in immunostimulation.<sup>32</sup> The identification of the compounds responsible for the immunostimulatory capacity of *B. clausii* probiotic strains is currently under investigation.

In conclusion, our results suggest that the beneficial clinical effects observed for Enterogermina can at least be partially the result of the antimicrobial and immunomodulatory activities of the *B. clausii* probiotic strains. These observations provide insights into the mechanisms responsible for *Bacillus* probiotic effects on the host and should encourage further study to understand the physiology of the interaction of these bacteria with the host and the role of different compounds produced by probiotic microorganisms.

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