Biochemical and Molecular Roles of Nutrients

Bacteria Used for the Production of Yogurt Inactivate Carcinogens and Prevent DNA Damage in the Colon of Rats\(^1,2\)

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ABSTRACT Lactic acid-producing bacteria prevent carcinogen-induced preneoplastic lesions and tumors in rat colon. Because the mechanisms responsible for these protective effects are unknown, two strains of lactic acid bacteria, Lactobacillus delbrueckii ssp. bulgaricus 191R and Streptococcus salivarius ssp. thermophilus CH3, that are used to produce yogurt, were investigated in vitro and in vivo to elucidate their potential to deactivate carcinogens. Using the “Comet assay” to detect genetic damage, we found that L. bulgaricus 191R applied orally to rats could prevent 1,2-dimethylhydrazine–induced DNA breaks in the colon in vivo, whereas St. thermophilus CH3 were not effective. However, in vitro, both strains prevented DNA damage induced by N-methyl-N-nitro-N-nitrosoguanidine (MNNG) in isolated primary rat colon cells. Extracts prepared from milk fermented with St. thermophilus CH3 were as efficient in deactivating MNNG as was L-cysteine. Isolated metabolites arising from bacteria during fermentation in the colon or in milk ([\(\pm\)] lactate, \((\pm)\) lactate, palmitic acid and isopalmitic acid) were not effective. We postulate that thiol-containing breakdown products of proteins, via catalysis by bacterial proteases, could be one mechanism by which MNNG or other carcinogens are deactivated in the gut lumen resulting in reduced damage to colonic mucosal cells.


KEY WORDS: • antigenotoxicity • rat colon cells • Comet assay • lactic acid bacteria

Fermented milk products are associated with several human health benefits. In addition to being palatable and nutritious, certain strains of lactic acid bacteria (LAB)\(^4\) help to maintain a well-balanced microflora. This positive influence on the ecology of the gut may contribute to alleviation of diarrhea, increased lactose tolerance in susceptible individuals and modulation of the immune response, as has been shown in human intervention trials (Bartram et al. 1994, Jiang et al. 1996, Kaila et al. 1992, Marteau et al. 1997). Some epidemiologic studies showed a decreased incidence of colon cancer in people consuming fermented milk products or yogurt (Malhotra 1977, Peters et al. 1992, Young and Wolf 1988), whereas other studies either showed no effect or even pointed to an increased risk (Kampman et al. 1994, Kearney et al. 1996).

This protective effect of fermented milk is supported by some model experiments in vitro or in animals that suggest the potential of certain LAB to inactivate carcinogens, thus prof-...

\(^1\) Supported in part by ECAIR-CT92-0256.
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\(^4\) Abbreviations used: BW, body weight; DMH, 1,2-dimethylhydrazine; IL, image length; LAB, lactic acid bacteria; MNNG, N-methyl-N-nitro-N-nitrosoguanidine; MRS, De Man, Rogosa and Sharpe broth.
We also investigated LAB metabolites, expected to occur as fermentation products in the gut lumen, for their ability to deactivate the genotoxic/carcinogenic compounds, MNNG, and hydroxyperoxide (H$_2$O$_2$). MNNG, a direct-acting carcinogen with alkylating properties, was chosen as a representative compound for endogenously formed N-nitroso compounds. In 1996, Bingham et al. showed that a diet high in meat could induce a threefold increase of endogenous N-nitroso compound production in humans.

**MATERIALS AND METHODS**

**Chemicals and bacterial strains.** N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) from Aldrich (Steinheim, Germany) was dissolved in physiological saline solution for the in vitro studies. 1,2-Dimethylhydrazine (DMH) was obtained from Sigma (Deisenhofen, Germany) and dissolved in physiological saline solution for application to rats in the in vivo studies. Hydrogen peroxide (H$_2$O$_2$) as a 30% solution was obtained from Merck (Darmstadt, Germany) and was diluted to 100 μmol/L l-Cysteine, t(+) lactate, t(−) lactate, palmitic acid and isopalmitic acid were from Sigma. LAB cultures were obtained from W. E. Sandine, Oregon State University, (St. thermophilus CH3 and L. bulgaricus 191R), or isolated from a commercially available yogurt from Germany (L. acidophilus). The other remaining St. thermophilus strains, A, B, C as well as L. bulgaricus A, B, C, were coded and provided by L. Lievense from Unilever, in the Netherlands. De Man Rogosa and Sharpe broth (MRS) from Merck was used as the growing medium for LAB. All LAB were inoculated into MRS broth and incubated at 25°C for 16 h. The resulting stationary phase cells were centrifuged (at 11,000 × g for 10 min) and the pellets were resuspended in saline solution to yield 10$^{12}$ cells/L with the use of a photometric calibration curve (Pool-Zobel et al. 1993a and 1996). Paracoccus demitricans was obtained from E. Mayer-Miebach (Institute for Process Engineering, Federal Research Centre for Nutrition, Karlsruhe, Germany) and grown in a modified mineral salt solution (Mayer-Miebach and Schubers 1991). All bacteria were applied intragastrically to rats in physiological saline solution at 10$^{12}$ cells/L, 10 mL/kg body weight (BW) in the morning on four consecutive days.

For the in vitro assays, bacterial cultures were grown in MRS, centrifuged and resuspended in fresh MRS, and preincubated with MNNG for 60 min (see in vitro assays).

**Acetone extracts.** Acetone extracts of LAB cultures, grown individually or together in milk (St. thermophilus CH3 and L. bulgaricus 191R), were prepared to elucidate whether the strains would cause the same type of protective activity during production of yogurt as they did when grown in MRS. The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Pool-Zobel et al. 1993a and 1996). The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Pool-Zobel et al. 1993a and 1996). The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Pool-Zobel et al. 1993a and 1996). The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Pool-Zobel et al. 1993a and 1996). The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Pool-Zobel et al. 1993a and 1996). The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Pool-Zobel et al. 1993a and 1996). The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Pool-Zobel et al. 1993a and 1996). The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Pool-Zobel et al. 1993a and 1996). The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Pool-Zobel et al. 1993a and 1996). The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Pool-Zobel et al. 1993a and 2017).
per animal or per in vitro concentration) were scored for the parameter “image length” (IL) using interactive image analysis (Perceptives Instruments, Surrey, UK). The images were grouped into classes of different degrees of damage: percentage of cells with DNA < 40 μm, 40–80 μm, 80–120 μm and > 120 μm. In analogy to the “arbitrary units” of Collins et al. (1995), similar units based on the length of images were used for evaluating the results and to weight the degree of DNA damage (image length units; IL-units). Therefore the percentage of cells with DNA 40–80 μm were multiplied by the factor 2, those with 80–120 μm by the factor 3 and > 120 μm by the factor 4, and were added to yield the IL-unit of each treatment group. This value was then used to compare these data with other published data.

Additionally, 50 cells of each treatment group were analyzed using the automatic version of the Comet assay image analyzer. The parameters, percentage of tail intensity (beginning at the center of the comet head) and the tail moment (a function of comet length and tail intensity), were scored.

Statistical analyses. All results are expressed as means ± SD (n as indicated). Comparisons were done with the statistical software package (Instat, GraphPad Prism, San Diego, CA). Differences in treatment groups were evaluated by one-way ANOVA followed by a multiple comparison vs. the positive control by the Dunnett post-test. Differences were considered significant at P < 0.05 and very significant at P < 0.01. In the study of palmitic and isopalmitic acids, a two-way ANOVA was performed, with dose of MNNG and palmitic acid or isopalmitic acid as factors tested.

RESULTS

Figures 1 and 2 show in vivo and in vitro effects of LAB in comparison to published data of related strains of different sources by Pool-Zobel et al. (1996), as well as that of an unrelated bacterium, P. denitrificans, introduced as a control for nonspecific bacterial action. For the data generated in vivo, it is evident that L. bulgaricus 191R is antigenotoxic in the DMH-treated rats, whereas St. thermophilus CH3 is not. In contrast, to St. thermophilus CH3, St. thermophilus A was antigenotoxic as were L. bulgaricus A and L. bulgaricus 191R (Fig. 1). The average of all strains tested here (first bar) as negative control showed a significant difference from the positive DMH control. Previous studies revealed no differences after LAB application in NaCl compared with NaCl application. Image lengths were 26.93 ± 4 μm for L. acidophilus (n = 3), 29.9 and 23.1 for L. gasseri, 23.2 and 29.0 for St. thermophilus and 25.1 and 23.7 for L. confusus in comparison to 26.01 ± 3 μm for NaCl (n = 7).

The protective effect of L. bulgaricus 191R was prevalent not only for the parameter IL-units (Fig. 1B) but also for the parameter percentage of cells with DNA < 40 μm (data not shown). The value of percentage of cells with DNA < 40 μm was 51.03 ± 27 for L. bulgaricus 191R (P = 0.026, n = 6) in comparison to 16.32 ± 11 (n = 10) for the DMH group. Relative to this percentage of undamaged cells, other strains were also protective with values of 46 ± 7 (P = 0.042, n = 4) for P. denitrificans and 76.59 ± 6 (P < 0.0001, n = 4) for L. acidophilus. The latter strain was included in this system as a control for antigenotoxic activity, because it inhibited the DNA-damaging effect of MNNO and DMH in previous studies. Heat-treatment of L. acidophilus resulted in a loss of protection (Pool-Zobel et al. 1993a and 1996).

Corresponding in vitro studies were performed with active intact cells of LAB cultures supplemented with fresh MRS for 80 min to elucidate whether the offer of nutrients supports the formation of antigenotoxic metabolites, similar to those suggested as being protective factors of L. acidophilus (Pool-Zobel et al. 1996). As shown in Figure 2, in vitro St. thermophilus CH3 and L. bulgaricus 191R were effective in reducing the genotoxic effect of MNNG (6.8 μmol/L) in colon cells as well.

In comparison, only one of the other three strains of St. thermophilus, St. thermophilus A, and two of the L. bulgaricus strains, L. bulgaricus B and C showed significant protection. The reduced genotoxicity achieved by treating the cells with St. thermophilus CH3 and L. bulgaricus 191R was apparent for the parameter IL-units (shown in the figures) as well as for the percentage of cells with DNA < 40 μm (data not shown). The supernatant of the active cells (incubated in fresh medium) of St. thermophilus CH3 increased the degree of intact colon cells (percentage of cells with DNA < 40 μm) from 38 ± 1 (MNNO + NaCl) to 53 ± 1 (MNNO + supernatant; P < 0.01, n = 3). In the same way, the supernatant of active cells of L. bulgaricus 191R resulted in a higher value of undamaged cells after MNNO-induced genotoxicity (47 ± 6, MNNO + supernatant vs. 33 ± 6 for MNNO + NaCl; P < 0.05, n = 3), whereas the supernatant of active cells of P. denitrificans, an unrelated bacterium, showed no effect (27 ± 4, MNNO + supernatant vs. 30 ± 6 for the MNNO + NaCl-control).

Table 1 shows the results of several experiments on the acetone extracts (10 and 20 μL/mL) of fermented milk against
The bars represent the mean values of isolated colon cells treated with preincubation mixture of various Paracoccus denitrificans (MRS) (data not shown). Colon cells pretreated with 195 μmol/L palmitic acid before 100 μmol/L H2O2 treatment of 15 min were not protected. The tail moment of cells pretreated with palmitic acid (7.17 ± 1.52) was not different from that of cells treated with H2O2 alone (7.08 ± 2.48).

**DISCUSSION**

Increasing public awareness of the probiotic concept of LAB is being promoted by the commercial propagation of LAB added to milk products, not to achieve fermentation in the traditional sense, but for the putative health-related benefits (Roberfroid et al. 1995). The health benefits claimed include well-being and stimulation of immune defense. In Germany, there has been considerable interest in these products, many of which have become available only recently. In light of these presumed health benefits, it is expected that consumption of LAB may increase in the near future. In addition, because probiotic food products are expected to contain considerably higher levels of LAB than traditionally fermented yogurts, and because these are especially selected to survive the gastrointestinal passage, higher levels of LAB in the gut may result. This assumption is supported by human studies showing that the daily intake of fermented milk products with living LAB, especially probiotic strains, resulted in a measurable increase in their number and metabolic activity in the colon (Link-Amster et al. 1994). Furthermore, a more aware intake of

10.2 μmol/L MNNG-induced genotoxicity in comparison to treatment with unfermented milk. Only fermentation by St. thermophilus CH3, 20 μL/mL, resulted in a reduction of IL-units, thus reflecting a reduction in the degree of DNA damage. No prevention of damage represented by an increase of the percentage of cells with DNA < 40 μm was achieved (data not shown). Because unfermented milk extract has no effect on MNNG (in comparison to MNNG treatment alone), the protective effect of St. thermophilus extract could be due to fermentation. Extract from yogurt made from milk fermented by both strains and extract of L. bulgaricus 191R milk were not protective.

The next question we addressed was to assess whether suggested individual metabolites produced during fermentation (either in milk or in the gut) could be antigenotoxic. Neither palmitic acid nor isopalmitic acid (97.5 μmol/L), which may result from lipolysis of milk during fermentation, inhibited the DNA-damaging effects of MNNG (10.2 or 17 μmol/L) in the Comet assay (Table 2). Two metabolites of LAB protein and carbohydrate metabolism, cysteine and lactate, were also tested for their ability to inactivate MNNG before exposure to colon cells (Fig. 3). At the two highest doses tested, cysteine inactivated MNNG, whereas L(+) lactate and D(−) lactate were not protective.

In the treatment schedule with H2O2-induced DNA damage and L(+) lactate, no protective effects were seen. On the contrary, an increase in DNA damage in H2O2-pretreated colon cells was observed at high lactate concentrations (results not shown). Colon cells pretreated with 195 μmol/L palmitic acid before 100 μmol/L H2O2 treatment of 15 min were not protected. The tail moment of cells pretreated with palmitic acid (7.17 ± 1.52) was not different from that of cells treated with H2O2 alone (7.08 ± 2.48).

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment of colon cells</th>
<th>Extract 10 μL/mL</th>
<th>Extract 20 μL/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>24 ± 4**</td>
<td>25 ± 13**</td>
</tr>
<tr>
<td>Extract of unfermented milk</td>
<td>17 ± 13**</td>
<td>34 ± 16**</td>
</tr>
<tr>
<td>MNNG, 10.2 μmol/L</td>
<td>189 ± 7</td>
<td>196 ± 21</td>
</tr>
<tr>
<td>MNNG, 10.2 μmol/L + extract of unfermented milk</td>
<td>144 ± 53</td>
<td>191 ± 33</td>
</tr>
<tr>
<td>MNNG, 10.2 μmol/L + extract of milk fermented with St. thermophilus CH3</td>
<td>128 ± 31</td>
<td>159 ± 49**</td>
</tr>
<tr>
<td>MNNG, 10.2 μmol/L + extract of milk fermented with L. bulgaricus 191R</td>
<td>150 ± 16</td>
<td>185 ± 10</td>
</tr>
<tr>
<td>MNNG, 10.2 μmol/L + extract of yogurt</td>
<td>145 ± 16</td>
<td>176 ± 31</td>
</tr>
</tbody>
</table>

1 Genotoxicity was evaluated by the Comet assay. Image length (IL)-units as parameters of DNA damage were determined after in vitro treatment of 30 min with DMSO or unfermented milk (as negative controls), with MNNG alone (as positive control) or with MNNG and acetone extracts of milk fermented with Streptococcus thermophilus CH3, Lactobacillus bulgaricus 191R or both strains (yogurt extract).

2 Results are means ± SD, n = 3.

3 * P < 0.05; ** P < 0.01; different from MNNG (positive control).

4 Results are means ± SD, n = 5.

5 ** P < 0.05; different from MNNG + unfermented milk.

**FIGURE 2** In vitro antigenotoxic activities of various strains of Streptococcus thermophilus and Lactobacillus bulgaricus against N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in the Comet assay. Image length (IL) units as parameters of DNA damage were determined in isolated colon cells of rats treated for 30 min with DeMan, Rogosa and Sharpe broth (MRS) (negative control), MNNG preincubated in MRS, 6.8 μmol/L (positive control), or with the preincubation mixture of MNNG together with several lactic acid bacteria (LAB) strains in MRS. Values are means ± SD; * P < 0.05 or ** P < 0.01 vs. positive control. The bars represent the mean values of isolated colon cells treated with either of the following: (A) MRS (n = 15), or MNNG preincubated in MRS (n = 15) or the preincubation mixture of various St. thermophilus (St. thermophilus A, B, C, CH3) or Paracoccus denitrificans (P. denitrificans (P.den.) and MNNG (n = 3); (B) MRS (n = 15), or MNNG preincubated in MRS (n = 15) or the preincubation mixture of various L. bulgaricus (L.b. A, B, C, CH3) of P. denitrificans (P.den.) and MNNG (n = 3).
TABLE 2

<table>
<thead>
<tr>
<th>Treatment of colon cells</th>
<th>Viability %</th>
<th>% cells with DNA3 damage</th>
<th>IL-units3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid, 97.5 μmol/L</td>
<td>94 ± 2</td>
<td>81 ± 1 **</td>
<td>39 ± 3 **</td>
</tr>
<tr>
<td>MNNG, 10.2 μmol/L</td>
<td>93 ± 2</td>
<td>21 ± 12</td>
<td>159 ± 25</td>
</tr>
<tr>
<td>MNNG, 10.2 μmol/L + PA</td>
<td>93 ± 2</td>
<td>31 ± 12</td>
<td>178 ± 25</td>
</tr>
<tr>
<td>MNNG, 17 μmol/L</td>
<td>94 ± 2</td>
<td>2 ± 2</td>
<td>205 ± 10</td>
</tr>
<tr>
<td>MNNG, 17 μmol/L + PA</td>
<td>95 ± 1</td>
<td>6 ± 6</td>
<td>189 ± 13</td>
</tr>
<tr>
<td>Isopalmitic acid, 97.5 μmol/L</td>
<td>92 ± 3</td>
<td>78 ± 7 **</td>
<td>47 ± 15</td>
</tr>
<tr>
<td>MNNG, 10.2 μmol/L</td>
<td>96 ± 1</td>
<td>10 ± 2</td>
<td>189 ± 2</td>
</tr>
<tr>
<td>MNNG, 17 μmol/L</td>
<td>95 ± 2</td>
<td>10 ± 7</td>
<td>183 ± 13</td>
</tr>
<tr>
<td>MNNG, 17 μmol/L + isoPA</td>
<td>95 ± 2</td>
<td>0 ± 0</td>
<td>211 ± 5</td>
</tr>
<tr>
<td>MNNG, 17 μmol/L + isoPA</td>
<td>94 ± 3</td>
<td>1 ± 2</td>
<td>208 ± 2</td>
</tr>
</tbody>
</table>

1 Genotoxicity was evaluated by the Comet assay. Undamaged DNA (% cells with DNA < 40 μm) and image length (IL) units as parameters of DNA damage were determined after in vitro treatment. 2 Results are means ± so from three independent experiments. 3 ** P < 0.01 from MNNG (positive control, 10.2 or 17 μmol/L).

Colon cancer is the second most common diet-dependent cancer in Western industrialized countries (Levi et al. 1995). On the basis of the knowledge that cancer induction is a complex multistep process initiated by the accumulation of mutations in tumor-suppressor genes and in protooncogenes, studying protective effects by measuring DNA damage in primary cells with the Comet assay is more relevant than performing other standard short-term genotoxic assays in bacteria or cultivated cells (Pool-Zobel 1996). At the moment, there is no direct evidence that LAB can inhibit carcinogenesis in humans. Most of the data postulating the protective effect of LAB are based on animal studies. However, these experiments emphasize that LAB can influence several mechanisms possibly linked to carcinogenesis such as preventing mutations, binding mutagens, inhibiting bacterial enzymes that form carcinogens from procarcinogens in the colon, decreasing pH in the colon lumen or activating the immune system. In humans, a decrease in fecal mutagenicity was revealed after consumption of L. acidophilus fermented milk together with fried meat (Lidbeck et al. 1992).

In this study, we have examined the protective effects of isolated LAB used to ferment yogurts in a "traditional" manner by investigating their antigenotoxic activity in the Comet assay and found a clear cut protection against DMH-induced damage in vivo with L. bulgaricus 191R. Protection by this strain was achieved by reducing the number of damaged colon cells as well as by reducing the degree of damage. In addition, P. denitrificans was able to increase the percentage of undamaged cells (percentage of cells with DNA < 40 μm); thus protection seems not to be restricted to LAB.

In contrast to the yogurt strain L. bulgaricus 191R, St. thermophilus CH3 was not protective in vivo. But both bacteria were effective in producing transient, antigenotoxic metabolites, which can deactivate MNNG in vitro. Thus, they acted like blocking agents, which prevent the initiation process of carcinogenesis (Wattenberg 1985). The underlying mechanism of the unknown metabolites could either be due to a binding of reactive intermediates before they reach the DNA or to an activation of enzymes that detoxify MNNG. In comparison to other strains of St. thermophilus and L. bulgaricus selected from other sources (over half of which had no antigenotoxic activity), they scored as LAB with good potential to be protectants. However, in these studies they were isolated and tested directly as viable LAB in culture, which means that no assumptions can be made concerning the protective activities that may be exerted by yogurt products. Such insight would require knowing the numbers of viable cells in the product and the lowest quantities necessary to achieve antigenotoxic effects in vivo (in humans).

However, studies with acetone extracts of LAB cultures in fermented milk show that if viable St. thermophilus were contained in the milk product, protective ingredients would be contained as well. It is possible that weak protective constituents of milk were converted to potent protective metabolites by the fermenting organisms. Nadathur et al. (1996) previously showed that free palmitic acid and isopalmitic acid, in particular, which may result from lipolysis of milk, were antimutagenic towards MNNG in the S. typhimurium mutagenicity assay. However, neither fatty acid was antigenotoxic against MNNG. Other protective ingredients of the fermented milk could include major products such as cysteine. We have shown here that cysteine may deactivate MNNG. Specific mechanisms of cysteine interaction with MNNG, potentiating its decomposition, are known (Lawley 1990). This deactivation in the colon lumen, resulting in less available carcinogen reaching the colon cells, is a plausible mechanism that may lead to cancer prevention in the colon (at least cancer induced by compounds such as MNNG). Several studies revealed an increase of cysteine as a consequence of the proteolytic system of LAB (Grudzinshaya and Koroleva 1970, Law and Kolstad 1983). The proteolytic activity will probably not be specific for yogurt bacteria because we have also shown other LAB to be antigenotoxic (Pool-Zobel et al. 1996). In addition, it will not...
be a “typical” feature of all LAB because we have shown that not all are protective. It would appear that protective activities are dependent on the survival of LAB through the intestinal tract, the accumulation of these organisms in the colon and their ensuing metabolic activities. Other compounds potentially associated with inducing genotoxicity in human colon cells as described by Pool-Zobel and Leucht (1997) should now be studied for their interaction with LAB and LAB metabolites in a similar manner. These studies will give us more in-depth knowledge concerning the total detoxifying capacities of these important and beneficial bacteria.

ACKNOWLEDGMENTS

The authors thank Martin Knoll, Renate Lambertz and Sudarshan R. Nadathur for excellent technical assistance.

LITERATURE CITED


Bingham, S. A., Pignatelli, B., Pollock, J. R. A., Ellul, A., Malaveille, C., Gross, G., Bartram, H. P., Scheppach, W., Gerlach, S., Ruckdeschel, G., Kelber, E. & Rittig, S. (1996) These studies will give us more in-depth knowledge concerning the total detoxifying capacities of these important and beneficial bacteria.


