

# Production of a Broad Spectrum Antimicrobial Substance by *Lactobacillus reuteri*

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Received 28 April 1988; revised 7 December 1988

*Lactobacillus reuteri* resides in the gastrointestinal tract of humans, swine, poultry and other animals. Resting cells of this species convert glycerol into a potent, broad-spectrum antimicrobial substance termed reuterin. Reuterin is a low molecular weight, neutral, water soluble compound, capable of inhibiting growth of species representing all bacterial genera tested thus far, including: *Escherichia*, *Salmonella*, *Shigella*, *Proteus*, *Pseudomonas*, *Clostridium* and *Staphylococcus*. Also affected, but to a lesser degree, are lactic acid bacteria belonging to the genera *Streptococcus*, *Pediococcus*, *Leuconostoc*, and *Lactobacillus*. In this report we describe a method to screen lactobacilli for production of unique antimicrobial substances and the discovery of reuterin.

KEY WORDS—*Lactobacillus reuteri*; Antimicrobial substance; Reuterin; Broad-spectrum activity.

## INTRODUCTION

A number of *Lactobacillus* species, including residents in the gastrointestinal ecosystem of humans and other animals, produce bacteriocidal proteins termed bacteriocins.<sup>1</sup> It has long been suspected, but to date not definitively proven, that these lactobacilli are also capable of producing unique, low molecular weight antimicrobial substances. The search for such novel substances produced by either homo- or heterofermentative species is necessarily complicated by their accumulation of metabolic end-products such as acetic and lactic acids (and hydrogen peroxide under certain conditions) which are strong antimicrobial agents in their own right. This is particularly true in the relatively low pH environments associated with lactobacilli, where these acids exist in their more potent undissociated forms. This search may be further hindered by screening procedures which ignore the possibilities that production of antimicrobial substances in some cases may require interaction between the 'producer' cells and the 'victim' cells and/or that only resting rather than growing cells are capable of producing these substances.

In this report we describe a screening procedure which circumvents some of these problems.  $\beta$ -galactosidase synthesis in *Escherichia coli* can be

induced under a variety of conditions with isopropyl- $\beta$ -D-thiogalactoside (IPTG) and measured with accuracy and sensitivity using O-nitrophenyl- $\beta$ -D-galactoside (ONPG) as a chromogenic substrate.<sup>2</sup> Inasmuch as lactobacilli respond poorly or not at all to IPTG induction,<sup>4</sup> we reasoned that co-culturing lactobacilli to be tested with *E. coli* cultures actively engaged in IPTG-induced  $\beta$ -galactosidase synthesis, could provide a rapid, sensitive method to screen the former for production of antimicrobial substances. Resting or growing lactobacilli in contact with 'victim' cells could thus be tested. Any decrease in  $\beta$ -galactosidase synthesis in these co-cultures would signal potential antimicrobial activity by the lactobacilli.

We also report the discovery of reuterin—a broad-spectrum antimicrobial substance produced by *L. reuteri* which is a prominent member of the *Lactobacillus* population in the gastrointestinal (GI) tract of humans, swine, poultry and other animals.<sup>5,6</sup>

## MATERIALS AND METHODS

### *Strains and Media*

*Lactobacillus* strains freshly isolated from pig intestine,<sup>1,12</sup> type strains of *L. reuteri* (*L. reuteri*

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DSM 20016 and *L. reuteri* ATCC 23273) and bacterial species used to determine the broad-spectrum activity of reuterin were obtained from the collection at the Department of Microbiology, Swedish University of Agricultural Sciences (Uppsala, Sweden). The *E. coli* strains were obtained from the collection at the Department of Microbiology, North Carolina State University (Raleigh, N.C., USA). *E. coli* strains YMC9 and 705 (glycerol negative, *glpK:Tn10* deletion mutants) and their respective isogenic parental strains, MC4100 and 707, were made available by E.C.C. Lin (Harvard University, USA).

The lactobacilli were maintained and grown in *Lactobacillus* carrying medium (LCM)<sup>3</sup> containing 20 mM glucose and enumerated using *Lactobacillus* selection medium (LBS) as previously described.<sup>11</sup> The *E. coli* cultures were grown in basal medium (BM) containing (g/L): vitamin-free casein hydrolysate (Difco), 3; ammonium citrate, 1.9; citric acid, 0.63; KH<sub>2</sub>PO<sub>4</sub> (anhyd), 12.6; MgSO<sub>4</sub> (heptahydrate), 0.2; pH adjusted to 7.0 with NaOH. After sterilisation, glycerol (40 mM) or other carbon sources were added as indicated. When used, filter sterilised IPTG (Sigma) was added at a final concentration of 50 mM and catalase (Sigma) at a final concentration of 500 units/ml. Viable counts of *E. coli* were obtained using MacConkey agar (Difco). Other bacteria in this study were grown either in Mueller-Hinton broth (Difco) or Thioglycolate broth (Difco) as indicated. All incubations were conducted at 37°C.

#### Culture conditions and assay procedures

All cultures were grown at 37°C either semi-anaerobically (i.e. 10 ml static cultures in screw-cap tubes) or aerobically by shaking 10 ml cultures in 50 ml Erlenmeyer flasks.  $\beta$ -galactosidase assays were performed as previously described.<sup>2</sup> *E. coli* cell dry weight measurements were calculated on the basis of absorbancy ( $A_{420\text{nm}}$ ) using a previously prepared standard curve relating  $A_{420}$  values to cell dry weight.<sup>2</sup> Susceptibility of various bacterial strains to reuterin present in filter-sterilised culture supernatant fractions was determined as follows: 1 ml of a supernatant fraction (or dilutions thereof) containing activity was added to 4 ml of Muller-Hinton or Thioglycolate broth and inoculated (along with control cultures) with the indicated bacterial strains at approximately 10<sup>6</sup> CFU/ml. The cultures were examined for visible growth after 24 and 48 h.

#### Screening lactobacilli for production of antimicrobial substances

*E. coli* K12 was grown overnight in BM containing 40 mM glycerol (or other substrates as indicated), harvested by centrifugation, washed twice with physiological saline, re-suspended to a cell mass of 50 to 55  $\mu\text{g}$  dry weight/ml in 5 ml fresh BM containing 40 mM glycerol and grown under aerobic or still culture conditions for 2 h prior to addition of 50 mM IPTG. After IPTG addition, samples were removed periodically, centrifuged and assayed for  $\beta$ -galactosidase (units/ml) as previously described.<sup>2</sup> Lactobacilli to be screened for antimicrobial activity were grown overnight in LCM containing 20 mM glucose, washed twice with physiological saline and were added 60 min after IPTG addition at a concentration of ca. 10<sup>9</sup> CFU/ml. No *Lactobacillus* addition or addition of heat-killed (10 min at 100°C) lactobacilli served as controls in these experiments.

#### RESULTS

When the screening procedure described above was implemented using 40 mM glycerol as the carbon and energy source with and without *L. reuteri* 1063 addition, the results shown in Figure 1 were obtained. This strain was among those chosen for initial screening because of its interesting surface properties; it is self-agglutinating and has a high degree of hydrophobicity and adherence to pig epithelial cells.<sup>12</sup> It can be seen that addition of *L. reuteri* 1063 to the aerobic cultures had no effect on  $\beta$ -galactosidase synthesis, but when added to the still culture a strong inhibitory effect was observed. The same effect was obtained when the type strains of this species were tested, i.e., *L. reuteri* DSM 20016 and its sub-culture, *L. reuteri* ATCC 23273. This inhibitory effect was not obtained when these cells were heat killed or when live, type strains of the following homo- and heterofermentative lactobacilli were tested: *L. acidophilus*, *L. bulgaricus*, *L. helveticus*, *L. plantarum*, *L. brevis*, *L. cellobiosus* and *L. fermentum*. Also negative in this regard were freshly isolated (from swine intestine) lactobacilli strains: 1031, 1044, 1046, 1048, 1068 and 1073.<sup>1</sup> It should be noted that strains 1044, 1048, 1068 and 1073 are related to *L. reuteri* DSM 20016 in terms of DNA-DNA homology, but to a lesser degree than strain 1063.<sup>1</sup>

This inhibitory effect was examined further using a lower concentration of *L. reuteri* 1063 cells and by determining the effect on cell viability rather than

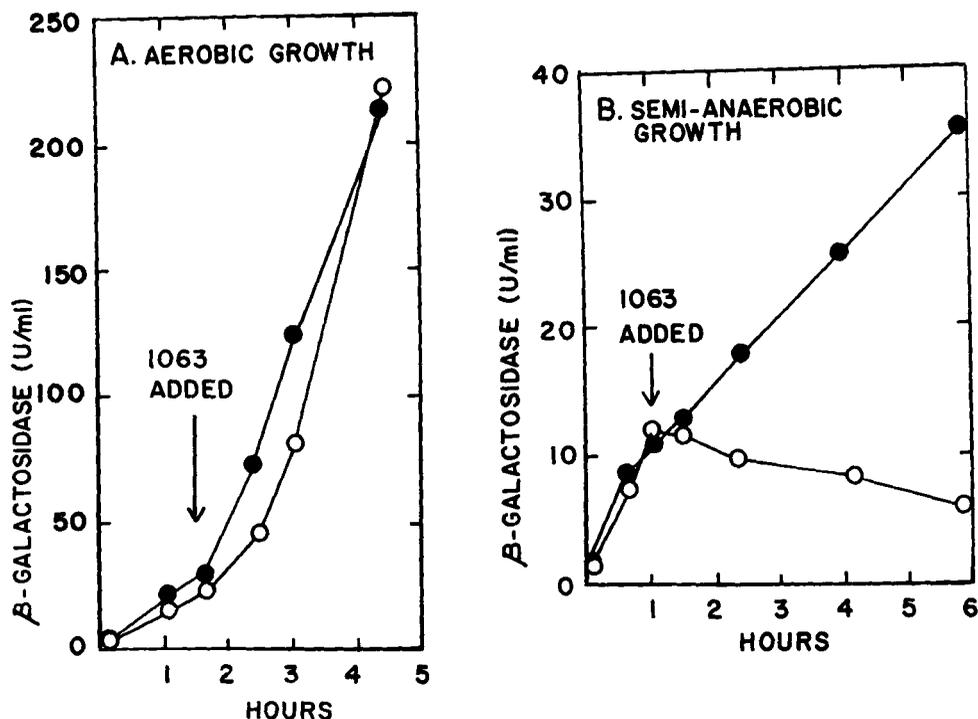


Figure 1. Effect of *L. reuteri* 1063 on  $\beta$ -galactosidase synthesis in *Escherichia coli* K12. The screening procedure described in Materials and Methods was used. At the time indicated by the arrow the *L. reuteri* 1063 cells were added (○) or omitted (●). One set of cultures was incubated aerobically (A) the other semi-anaerobically (B)

$\beta$ -galactosidase synthesis. The results of this experiment are shown in Figure 2. It can be seen that *L. reuteri* 1063 does not grow under these conditions but that after 4 h a bactericidal substance was produced which decimated the *E. coli* but not the *L. reuteri* population. It can also be seen that this effect did not occur when glycerol was omitted from the culture medium.

Antimicrobial activity in lactobacilli attributable to bacteriocins is well established.<sup>8</sup> In contrast, low molecular weight antimicrobial substances reportedly produced by lactobacilli generally turn out to be lactic acid fermentation products such as acetic and lactic acid and/or hydrogen peroxide.<sup>7</sup> The results reported in Table 1 show that this is not the case for reuterin. It can be seen from these data that reuterin production occurs in the absence of any significant change in the culture medium pH and in the presence of excess catalase activity. These data show reuterin to be produced and to function as a bactericide in the neutral pH range. This is in contrast to the recently reported antimicrobial substance derived from a human *Lactobacillus* strain which is active only between pH 3 and 5<sup>10</sup>. We know

that reuterin has a molecular weight of less than 200 g/mole; it is resistant to protease activity (unpublished observations) and therefore is not a bacteriocin.

A variety of substrates were examined for their ability to support reuterin production (Table 2). Some of these substances (i.e. glucose, mannose, fructose, mannitol, sorbitol, gluconate, xylose and glycerol) supported *E. coli* growth while others (i.e. ribitol, arabitol and glyceraldehyde) did not. It can be seen that only glycerol and glyceraldehyde (which presumably can be reduced to glycerol under these conditions) were effective in promoting reuterin production. The ability of *E. coli* cells to catabolise glycerol plays no role in reuterin synthesis. This was determined by showing that the *L. reuteri* 1063-*E. coli* co-cultures produced reuterin from glycerol whether parental strains (*E. coli* strains MC4100 and 707) or isogenic *glpK:Tn10* mutant strains (YMC9 and 705) were used; the latter being incapable of catabolising glycerol.

The glycerol requirement for reuterin production was further demonstrated by the data shown in Table 3. Among a number of phosphorylated and

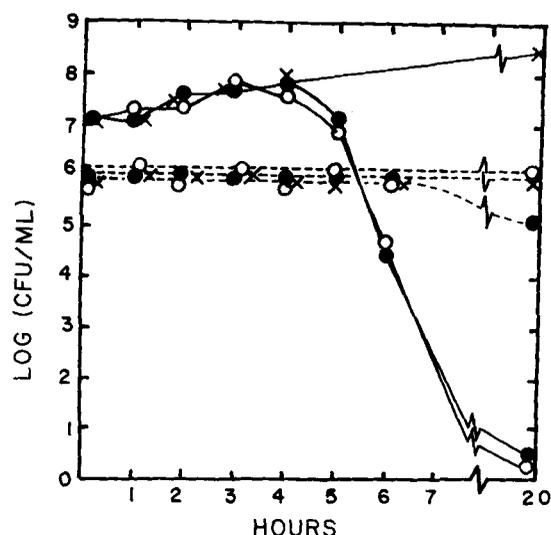


Figure 2. Effect of *L. reuteri* 1063 on viability of *Escherichia coli* K12. A co-culture containing  $\log_{10}$  6.0 CFU/ml *L. reuteri* cells and  $\log_{10}$  7.0 CFU/ml *E. coli* cells was incubated in BM (see Materials and Methods) containing the following carbon sources: BM supplemented with 40 mM glycerol (O), BM supplemented with 40 mM glycerol but eliminating the citrate and citric acid (●), and BM alone (X). The CFU/ml for *L. reuteri* (---) and *E. coli* (—) were estimated at the indicated times over a 20 h co-culture period.

Table 1. Reuterin is not an acidic end-product or hydrogen peroxide

| <i>L. reuteri</i> | Additions* | <i>E. coli</i> |                    | Percentage inhibition | pH† |
|-------------------|------------|----------------|--------------------|-----------------------|-----|
|                   |            | Catalase       | $\log_{10}$ CFU/ml |                       |     |
| +                 | —          | 7.2            | 5.3                | 99                    | 6.9 |
| +                 | +          | 7.2            | 5.3                | 99                    | 6.9 |
| —                 | —          | 7.2            | 8.3                | 0                     | 6.5 |
| —                 | +          | 7.2            | 8.4                | 0                     | 6.5 |

\*Co-cultivation conditions were used as described in Figure 2. The *L. reuteri* cells were grown overnight in LCM containing 20 mM glucose, washed twice with physiological saline and  $\log_{10}$  6.0 CFU/ml inoculated into BM containing 40 mM glycerol and the indicated number of *E. coli* cells with and without catalase as indicated.

†pH recorded after 6 h incubation; the initial pH was 7.0

non-phosphorylated three carbon substrates tested, only glycerol (and glyceraldehyde) were capable of promoting reuterin production. These experiments also showed that reuterin remains in the supernatant fraction after removing the cells by centrifugation.

Table 2. Effect of various carbon sources on reuterin production by *L. reuteri* 1063\*

| Substrate      | Additions         |                                     | Percentage inhibition |
|----------------|-------------------|-------------------------------------|-----------------------|
|                | <i>L. reuteri</i> | <i>E. coli</i> † $\log_{10}$ CFU/ml |                       |
| Glucose        | —                 | 8.1                                 |                       |
|                | +                 | 8.1                                 | 0                     |
| Mannose        | —                 | 7.8                                 |                       |
|                | +                 | 7.9                                 | 0                     |
| Fructose       | —                 | 8.3                                 |                       |
|                | +                 | 8.5                                 | 0                     |
| Mannitol       | —                 | 8.4                                 |                       |
|                | +                 | 8.4                                 | 0                     |
| Sorbitol       | —                 | 8.3                                 |                       |
|                | +                 | 8.3                                 | 0                     |
| Gluconate      | —                 | 8.5                                 |                       |
|                | +                 | 8.5                                 | 0                     |
| Xylose         | —                 | 7.8                                 |                       |
|                | +                 | 7.8                                 | 0                     |
| Ribitol        | —                 | 6.7                                 |                       |
|                | +                 | 6.7                                 | 0                     |
| Arabitol       | —                 | 6.4                                 |                       |
|                | +                 | 6.5                                 | 0                     |
| Glycerol       | —                 | 7.1                                 |                       |
|                | +                 | 4.7                                 | >99                   |
| Glyceraldehyde | —                 | 6.4                                 |                       |
|                | +                 | 4.5                                 | >99                   |

\*The co-culture conditions were as described in Table 1 except for addition of 40 mM concentrations of the indicated substrates. The initial *L. reuteri* 1063 and *E. coli* K12 cell numbers were  $\log_{10}$  6.0 and 6.2, respectively.

†Numbers present 6 h later

Reuterin-containing supernatant fractions thus obtained were used to determine the sensitivity of swine enteropathogenic *E. coli* strains and other Gram-negative as well as Gram-positive bacteria to reuterin (Table 4). These data show that all strains tested were sensitive to reuterin. It is unknown at this time why 3 to 5 fold higher concentrations of reuterin are needed to inhibit growth of the lactic acid group of bacteria (i.e. *Streptococcus lactis*, *Pediococcus cerevisiae*, *Leuconostoc mesenteroides*, *L. acidophilus* and *L. plantarum*) than are needed to inhibit the other bacteria tested.

## DISCUSSION

*L. reuteri* is a newly recognised species of *Lactobacillus*<sup>5,6</sup> which includes strains previously classified as *L. fermentum*. Both species are heterofermentors

ANTIMICROBIAL ACTIVITY OF *L. REUTERI*

Table 3. Production of reuterin from various three carbon substances

| Substrate*                | <i>E. coli</i> †<br>log <sub>10</sub> CFU/ml | Percentage inhibition |
|---------------------------|--|-----------------------|
| Pyruvate                  | 9.6  | 0                     |
| Phosphoenolpyruvate       | 9.6  | 0                     |
| Phosphoglycerate          | 9.5  | 0                     |
| β-glycerolphosphate       | 9.5  | 0                     |
| Dihydroxyacetonephosphate | 9.6  | 0                     |
| Glycerol                  | 5.6  | >99                   |
| Glyceraldehyde            | 5.8  | >99                   |

\*The co-culture conditions were essentially as described in Table 2 except for use of the indicated three carbon substrates at a 40 mM concentration. After 6 h incubation the cells were removed by centrifugation and 1 ml of each supernatant fraction was filter-sterilised and added to 9 ml of fresh BM containing 20 mM glucose and inoculated with *E. coli* at a concentration of log<sub>10</sub> 7.0 CFU/ml.

†Numbers present 6 h later

Table 4. Sensitivity of selected Gram-positive and Gram-negative bacteria to reuterin

| Species* (No.)                        | Sensitivity |
|---------------------------------------|-------------|
| <i>Escherichia coli</i> (7)†          | 20–25       |
| <i>Salmonella typhimurium</i> (1)     | 20          |
| <i>Pseudomonas aeruginosa</i> (2)     | 25          |
| <i>Staphylococcus epidermidis</i> (1) | 15          |
| <i>Bacillus megaterium</i> (1)        | 20          |
| <i>Streptococcus lactis</i> (2)       | 5           |
| <i>Pediococcus cerevisiae</i> (1)     | 5           |
| <i>Leuconostoc mesenteroides</i> (1)  | 4           |
| <i>Lactobacillus acidophilus</i> (6)  | 2–5         |
| <i>Lactobacillus plantarum</i> (2)    | 5           |

\*Sensitivity expressed as maximum dilution of a supernatant fraction containing reuterin prepared exactly as described in Table 3 from a co-culture incubated in the presence of glycerol. No pH adjustment of the supernatant fractions were necessary (see Table 1).

†Including 6 swine enteropathogens

and similar in their carbohydrate fermentation profiles, but *L. reuteri* differs in G & C content (39–41 mole per cent) of its DNA and has lysine as the peptidoglycan diaminoacid. It inhabits the GI tract of humans and other animals, and preliminary studies along this line (Pagano, Lecce and Dobrogosz, unpublished observations) have shown reuterin-

producing *L. reuteri* to be a prominent component of the *Lactobacillus* population associated with the proximal regions (stomach and small intestine) of the swine GI tract.

In this report we present evidence that in the presence of glycerol *L. reuteri* cells produce reuterin—a broad-spectrum antimicrobial substance. Preliminary studies on reuterin isolated by high performance liquid chromatography show that it is not an acid end-product, but rather that it is a neutral, water soluble, non-protein, low molecular weight (less than 200 g/mole) derivative of glycerol (Talarico and Dobrogosz, unpublished observations). Further purification and chemical characterisation analyses are underway.

Since the turn of the century<sup>9</sup> enteric lactobacilli have been thought to contribute to the host's health. A general belief has also persisted that these bacteria synthesise antimicrobial substances in addition to the end-products (i.e., lactic and acetic acids and hydrogen peroxide) associated with the various lactic acid fermentations. This has been confirmed with respect to production of bacteriocins which are proteins exhibiting antibacterial activity. A wide assortment of bacteriocins produced by lactobacilli, including enteric lactobacilli are known to exist.<sup>8</sup> With respect to production of non-protein, low molecular weight inhibitory substances the record is less clear. A number of such substances are reported in the literature, including the recent report by Silva *et al.*<sup>10</sup> To the best of our knowledge none have yet been rigorously identified in terms of their chemical composition; many have turned out to be nothing more than the traditional lactic acid fermentation end-products described above.<sup>7</sup>

## ACKNOWLEDGEMENT

These studies were supported by the Swedish Council for Forestry and Agricultural Research. Paper number 11560 of the journal series of the North Carolina Agricultural Research Service (Raleigh, NC 27695, USA). The *Lactobacillus reuteri*-reuterin system is protected by worldwide patent applications licensed to ProBiologics International, Inc., 8606 Jersey Court, Raleigh, NC 27612 (USA).

## REFERENCES

1. Axelsson L, Lindgren SE. (1987). Characterization and DNA homology of *Lactobacillus* strains isolated from pig intestine. *Journal of Applied Bacteriology* **62**, 433–440.
2. Dobrogosz WJ. (1981). Enzymatic activity, In Gerhardt P (ed) *Manual of Methods for General*

- Bacteriology*. American Society for Microbiology, Washington, DC, 365–392.
3. Efthymiou C and Hansen PA. (1962). An antigenic analysis of *Lactobacillus acidophilus*. *Journal of Infectious Diseases* **110**, 258–267.
  4. Hasan N and Durr IF. (1974). Induction of  $\beta$ -galactosidase in *Lactobacillus plantarum*. *Journal of Bacteriology* **120**, 66–73.
  5. Kandler O, Stetter KO, Kohl R. (1980). *Lactobacillus reuteri* sp. nov., a new species of heterofermentative lactobacilli. *Zentralblatt für Bakteriologie Mikrobiologie and Hygiene. T. Abt. Org.* **CI**, 264–269.
  6. Kandler O and Weiss N. (1986). Regular, non-sporing gram-positive rods. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) *Bergey's Manual of Systematic Bacteriology* Vol. 2, Williams and Wilkins Co Baltimore, 1208–1234.
  7. Klaenhammer, TR. (1982). Microbiological considerations in selection and preparation of *Lactobacillus* strains for use as dietary adjuncts. *Journal of Dairy Science* **65**, 1339–1349.
  8. Klaenhammer, TR. (1988). Bacteriocins of lactic acid bacteria. *Biochimie*, **70**, 337–349.
  9. Mechnikoff E. (1908). *The Prolongation of Life*. Arno Press, New York.
  10. Silva M, Jacobus NV, Denke C, Gorbach SL. (1987). Antimicrobial substance from a human *Lactobacillus* strain. *Antimicrobial Agents and Chemotherapy* **31**, 1231–1233.
  11. Shrago AW, Chassy BM, Dobrogosz WJ. (1986). Conjugal plasmid transfer (pAM $\beta$ 1) in *Lactobacillus plantarum*. *Applied and Environmental Microbiology* **52**, 574–576.
  12. Wadstrom T, Anderson K, Sydow M, Axelsson L, Lindgren SE, Gullmar B. (1987). Surface properties of lactobacilli isolated from the small intestines of pigs. *Journal of Applied Bacteriology*, **62**, 513–520.