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# Reuterin production by lactobacilli isolated from pig faeces and evaluation of probiotic traits

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## ABSTRACT

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**Aims:** To determine the production of reuterin by lactobacilli isolates from pig faeces and to evaluate their potential as probiotic bacteria.

**Methods and Results:** Twenty-eight of 165 lactobacilli isolates produced reuterin in the presence of glycerol. Six isolates yielding high levels of reuterin with respect to type strain *Lactobacillus reuteri* CECT 925T were identified as *Lact. reuteri*. They were able to survive at pH 3 and subsequent exposure to cholic acid or oxgall, and presented bile salt hydrolase and bacteriocin-like activities.

**Conclusions:** Reuterin production is a frequently found trait among lactobacilli isolated from pig faeces. Selected *Lact. reuteri* isolates were able to survive at conditions likely to be encountered throughout the gastrointestinal tract.

**Significance and Impact of the Study:** High yields of reuterin may be obtained from selected isolates of *Lact. reuteri*. Probiotic characteristics of isolates studied in the present work suggest their application in food and feed.

**Keywords:** *Lactobacillus reuteri*, antimicrobial, probiotics, reuterin.

## INTRODUCTION

*Lactobacillus reuteri* is a resident of the gastrointestinal tract of humans and animals, and appears to be the dominant heterofermentative species in this ecosystem. *Lactobacillus reuteri* and *Lact. acidophilus* group were found to be the major *Lactobacillus* species in the piglet intestine (Naito *et al.* 1995).

Reuterin ($\beta$-hydroxypropionaldehyde) is a broad spectrum antimicrobial compound produced by some strains of *Lact. reuteri* during anaerobic fermentation of glycerol (Axelsson *et al.* 1989). The enzyme responsible for the formation of $\beta$-hydroxypropionaldehyde is the co-enzyme B12-dependent glycerol dehydratase (Smiley and Sobolov 1962). It can be synthesized *in vitro* under pH, temperature and anaerobic conditions similar to those in the gastrointestinal tract (Chung *et al.* 1989). Reuterin was isolated, purified and identified as an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of $\beta$-hydroxypropionaldehyde (Talarico and Dobrogosz 1989). Its bactericidal activity against *Listeria monocytogenes* and *Escherichia coli* O157:H7 was observed in milk and cottage cheese by El-Ziney and Debevere (1998). Reuterin is water-soluble, active at a wide range of pH values and resistant to proteolytic and lipolytic enzymes, and it may be a suitable compound for food biopreservation.

The aim of the present work was to isolate reuterin-producing lactobacilli from pig faeces and to quantify reuterin production by these new isolates. Some of their probiotic characteristics, such as the ability to survive under gastrointestinal conditions, bile salt hydrolase activity and inhibitory activity of neutralized culture supernatants were also investigated.

## MATERIALS AND METHODS

### Isolation of reuterin-producing lactobacilli

Pig faeces (33 samples) were obtained from different farms in north-west Spain. Decimal dilutions were plated on Rogosa agar (Biolife Italiana S.r.l., Milan, Italy) and

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incubated anaerobically (AnaeroGen$^{TM}$ Oxoid, Unipath Ltd, Basingstoke, UK) at 37°C for 48 h.

Approximately six colonies per sample were selected and subcultured anaerobically in de Man Rogosa Sharpe (MRS) broth (Biolife) at 37°C for 24 h. Isolates ($CO_2$ producing) were inoculated in MRS broth with glycerol (250 mmol $l^{-1}$). After 24 h at 37°C in anaerobiosis, 1 ml of culture was centrifuged at 12 000 **g** for 5 min and 300 $\mu$l of supernatant were mixed with 150 $\mu$l of tryptophan solution (3 g $l^{-1}$ in 0·1 mol $l^{-1}$ HCl) and 600 $\mu$l of 35% HCl. The mixture was heated at 60°C for 5 min. $\beta$-Hydroxypropionaldehyde produced from glycerol was determined by dehydration to acrolein (Smiley and Sobolov 1962), developing a colour that confirms the presence of reuterin. Reuterin-producing isolates were purified, examined for Gram staining, catalase production, cellular morphology by phase contrast microscopy, and streaked on KF *Streptococcus* agar (Oxoid) and Rogosa agar.

Gram-positive, catalase-negative, rod-shaped isolates unable to grow on KF *Streptococcus* agar plates were selected for further studies. They were maintained as stock cultures frozen at −80°C in MRS broth with 15% glycerol and propagated twice before using in experiments.

## Quantification of reuterin

Reuterin production by isolates was quantified by the assay described previously with *Lact. reuteri* CECT 925T as positive control. Isolates were inoculated in MRS broth and incubated anaerobically at 37°C for 24 h. Pellets obtained after centrifugation of 1 ml of cultures at 7000 *g* for 5 min were washed, resuspended in 0·5 ml of water–glycerol solution (250 mmol $l^{-1}$) and incubated at 37°C for 3 h. Suspensions were centrifuged and reuterin was quantified in 100 $\mu$l of supernatant as described by Smiley and Sobolov (1962). Differences in absorbance measured at 490 nm in a Beckman DU 650 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA) accounted for differences in reuterin production when compared against acrolein standards.

## Inhibitory activity of reuterin

The inhibitory activity of reuterin in supernatants of water–glycerol solution was determined after a modification of the method described by Chung *et al.* (1989). *Escherichia coli* K12 was used as an indicator strain. Reuterin-containing supernatants were serially diluted and 150 $\mu$l were added to microtiter plate wells that were inoculated with 150 $\mu$l of an overnight culture of *E. coli* (at *ca* $10^4$ CFU $ml^{-1}$) in double strength Tryptic Soy Broth (TSB; Biolife). A solution of water–glycerol without reuterin was used as negative control. Plates were incubated at 37°C for 24 h. Growth was examined visually and arbitrary units (AU) were defined as the reciprocal of the highest dilution that did not permit growth of the indicator strain.

## Identification of selected reuterin-producing isolates

API 50 CHL strips (Biomérieux, Lyon, France) were used for the biochemical characterization of reuterin-producing isolates.

A polymerase chain reaction (PCR) method based on amplification of 16S RNA coding sequences of *Lact. reuteri* and *Lact. fermentum* described by Chagnaud *et al.* (2001) was used to identify selected isolates at species level, with primers pairs LOWLAC-REUT1 and LOWLAC-FERM1, and PCR conditions recommended by the authors. Genomic DNA from *Lact. reuteri* CECT 925T and *Lact. fermentum* CECT 4007T were used as control in both PCR reactions.

Preparation of genomic DNA was performed according to Ausubel *et al.* (1994) from 1·5 ml of each bacterial overnight culture after adding 10 mg $ml^{-1}$ lysozyme to the lysis buffer. DNA obtained was finally resuspended in 50 $\mu$l of TE (10 mmol $l^{-1}$ Tris–HCl, 1 mmol $l^{-1}$ EDTA) and stored at −20°C. One microlitre of a 1/10 DNA dilution was used as template for PCR reactions.

## PCR detection of glycerol dehydratase gene

A second PCR reaction was conducted using genomic DNA from the selected isolates to detect the presence of the glycerol dehydratase encoding gene involved in reuterin production, using primers GD1 and GD2 and PCR conditions described by Claisse and Lonvaud-Funel (2001). DNA from *Lact. reuteri* CECT 925T was used as positive control.

## Survival under gastric and intestinal conditions

*Lactobacillus reuteri* isolates were exposed to HCl at pH 3 and consecutively to cholic acid or oxgall (Oxoid) to simulate gastric and intestinal conditions in a two-step procedure based on Haller *et al.* (2001). *Lactobacillus reuteri* CECT 925T, *Lact. reuteri* B and *Lact. paracasei* S isolated from two commercial probiotic products and identified by molecular techniques (data not shown), and *Lactobacillus* PRO 244, a sensitive strain obtained from infant faeces, were also included. Lactobacilli were grown anaerobically in MRS broth at 37°C for 24 h, centrifuged at 7000 *g* for 5 min, washed with phosphate-buffered saline (PBS) pH 7·2 and resuspended in 1 ml PBS. An aliquot of 0·1 ml of the suspension was added to 0·9 ml HCl pH 3 and incubated at 37°C for 1 h. After incubation, two aliquots of 0·1 ml of this mixture were added to 0·9 ml oxgall (0·3% final concentra-

tion) or to 0·9 ml cholic acid (10 mmol $l^{-1}$ final concentration) (Sigma Chemical Co., St Louis, MO, USA), and incubated at 37°C for 1 h. Lactobacilli counts after treatments were determined by surface plating on MRS agar, which was incubated at 37°C for 24 h under anaerobiosis.

### Bile salt hydrolase activity

Bile salt hydrolase (BSH) activity was screened by spotting in duplicate 10 $\mu$l of cultures grown in MRS broth on the surface of MRS agar plates supplemented with 0·5% sodium salt of taurodeoxycholic acid (TDCA, Sigma) and 0·37 g $l^{-1}$ of $CaCl_2$ (Franz *et al.* 2001). Plates were incubated anaerobically at 37°C for 72 h. BSH was detected by the presence of precipitation zones below and around the colony.

### Antimicrobial activity in culture supernatants

Presence of bacteriocin-like antimicrobial activity was examined by an agar diffusion assay using *Enterococcus faecalis* EF, *Lact. buchneri* St2A, *L. monocytogenes* Ohio, *Lact. plantarum* CECT 4645, *Clostridium tyrobutyricum* NZ8 and *E. coli* K12 as indicator strains. Cells were grown anaerobically in MRS broth and centrifuged after incubation. Aliquots of 25 $\mu$l of neutralized supernatants were placed in duplicate into wells (5 mm diameter) performed on Tryptic Soy Agar (TSA; Biolife) plates seeded with an overnight culture of each indicator inoculated at 0·1%. After incubation at 37°C for 24 and 48 h, antimicrobial activity was detected by the presence of inhibition zones.

## RESULTS AND DISCUSSION

### Selection of reuterin-producing lactobacilli

*Lactobacillus reuteri* and other heterofermentative lactobacilli are prevalent in the healthy intestinal tract of humans and animals. Reuterin is an inhibitor of different undesirable bacteria, including enteric pathogens.

In the present work, 165 isolates from 33 samples of pig faeces were obtained from Rogosa agar plates. A total of 119 isolates produced gas under anaerobic conditions in MRS broth and were considered to be heterofermentative. They were inoculated in MRS broth supplemented with glycerol to evaluate their ability to produce reuterin in anaerobic conditions. Reuterin production was observed directly in the growth media of 28 isolates by a change in colour because of the acrolein formed by dehydration of $\beta$-hydroxypropionaldehyde (reuterin). This methodology provides a fast and simple tool for screening a high number of isolates for reuterin production.

The 28 reuterin-producing isolates were obtained from 10 different samples of pig faeces. They were Gram-positive, catalase-negative rods, able to grow on Rogosa agar under aerobic conditions and unable to grow on KF agar.

Differences in reuterin production in water–glycerol solution, measured as acrolein formation, were observed among the 28 reuterin-producing isolates (Table 1). Similar amounts of acrolein and inhibitory activity (AU) detected in some isolates from the same sample suggest that they correspond to the same strain. A low amount of acrolein, 0·64 $\mu$g $ml^{-1}$, was recorded for the type strain *Lact. reuteri* CECT 925T. Higher values of acrolein were obtained for all the isolates, ranging from 0·87 to 58·83 $\mu$g $ml^{-1}$. Presence of active reuterin was confirmed in the supernatants of water–glycerol solution using *E. coli* K12 as indicator (Table 1). A high inhibitory activity was generally observed in those isolates for which high levels of acrolein were recorded.

Six isolates, each from a different sample, producing reuterin at levels higher than 15 $\mu$g $ml^{-1}$ were selected for further studies.

**Table 1** Reuterin production by 28 lactobacilli isolates from pig faeces expressed as the amount of acrolein ($\mu$g $ml^{-1}$) formed from reuterin and as the inhibitory activity against *Escherichia coli* K12 in arbitrary units (AU)

| Isolate | Sample | Acrolein | AU |
|---|---|---|---|
| PRO 105 | P4 | 13·88 | 8 |
| PRO 107 | P4 | 34·32 | 16 |
| PRO 108 | P4 | 34·58 | 16 |
| PRO 109 | P5 | 46·42 | 32 |
| PRO 110 | P5 | 22·59 | 4 |
| PRO 111 | P5 | 44·93 | 32 |
| PRO 112 | P5 | 44·27 | 32 |
| PRO 113 | P5 | 43·05 | 32 |
| PRO 114 | P6 | 5·16 | 4 |
| PRO 116 | P6 | 53·43 | 16 |
| PRO 117 | P6 | 47·27 | 32 |
| PRO 118 | P6 | 49·94 | 32 |
| PRO 132 | P9 | 51·78 | 32 |
| PRO 133 | P9 | 58·83 | 64 |
| PRO 134 | P9 | 56·23 | 64 |
| PRO 135 | P9 | 47·94 | 32 |
| PRO 136 | P9 | 1·98 | 4 |
| PRO 137 | P10 | 37·94 | 8 |
| PRO 146 | P10 | 4·81 | 4 |
| PRO 147 | P20 | 20·83 | 16 |
| PRO 148 | P21 | 34·76 | 32 |
| PRO 149 | P21 | 38·62 | 32 |
| PRO 150 | P23 | 11·04 | 4 |
| PRO 151 | P25 | 0·87 | 4 |
| PRO 152 | P25 | 17·72 | 8 |
| PRO 153 | P26 | 12·24 | 4 |
| PRO 154 | P26 | 7·42 | 4 |
| PRO 155 | P26 | 4·04 | 4 |

## Identification of selected isolates

Selected reuterin-producing isolates were subjected to sugar fermentation pattern analysis using API 50 CHL test strips and to PCR amplification of 16S RNA coding sequences.

The fermentation profile obtained by API 50 CHL test strips for the six isolates led to their classification as *Lact. fermentum*, because of the inability to distinguish between *Lact. fermentum* and *Lact. reuteri*.

The amplification of a ±1000 bp fragment from the genomic DNA using the species-specific primer pair LOWLAC-REUT1 identified the isolates as *Lact. reuteri*.

## Detection of glycerol dehydratase gene

A 279 bp amplicon corresponding to a fragment of glycerol dehydratase gene sequence was obtained in the six isolates tested (Fig. 1). Glycerol dehydratase genes of lactobacilli isolated from spoiled ciders were detected only in strains with positive glycerol dehydratase activity (Claisse and Lonvaud-Funel 2001). Detection of glycerol dehydratase encoding gene in our isolates supported the ability of these *Lact. reuteri* strains to produce reuterin.

## Probiotic characteristics

Behaviour of *Lact. reuteri* under consecutive exposure to pH 3 and cholic acid or oxgall was investigated after incubation of cells at 37°C in HCl (pH 3) for 1 h and then in oxgall (0·3%) or cholic acid (10 mmol $l^{-1}$) for 1 h to simulate gastrointestinal transit. All six isolates tested were tolerant to pH 3 and to the subsequent stress of bile, with similar results in the presence of cholic acid or oxgall (Table 2). Similar results were obtained with *Lact. reuteri* CECT 925T and *Lact. reuteri* B, whereas a considerable decrease in counts of *Lact. paracasei* S was observed after pH 3-oxgall treatment. *Lactobacillus* PRO 244 was not detected after cholic acid or oxgall treatments. These results indicate that the isolates from pig faeces would survive *in vivo* the low pH of the stomach and the bile-rich environment of the intestine, as expected for commercial strains. Haller *et al.* (2001) compared the human isolate *Lact. johnsonii* La1 with some food isolates of *Lact. plantarum, Lact. sakei* and *Lact. paracasei* after consecutive exposure to pH 2·5 and bile acid. Food fermenting lactobacilli, mainly *Lact. plantarum*, presented similar tolerance to pH 2·5 than *Lact. johnsonii*. *Lactobacillus reuteri* BSA 131, a strain able to grow at pH 2 and resistant to bile salts, was assessed as probiotic in piglets by Chang *et al.* (2001).

**Table 2** Counts (log CFU $ml^{-1}$) of six selected *Lactobacillus reuteri* isolates, a reference strain, two commercial strains and a sensitive isolate, before (0 h) and after consecutive exposure to pH 3 for 1 h and cholic acid or oxgall for 1 h, at 37°C

| Isolate | 0 h | pH 3 | Cholic acid | Oxgall |
|---|---|---|---|---|
| PRO 108 | 9·10 | 8·76 | 8·54 | 8·48 |
| PRO 109 | 9·03 | 8·65 | 8·48 | 8·54 |
| PRO 133 | 9·00 | 8·70 | 8·46 | 8·36 |
| PRO 137 | 9·13 | 8·80 | 8·60 | 8·54 |
| PRO 149 | 9·16 | 8·72 | 8·56 | 8·37 |
| PRO 152 | 9·13 | 8·80 | 8·51 | 8·59 |
| *Lact. reuteri* CECT 925T | 9·37 | 8·87 | 8·76 | 9·01 |
| *Lact. reuteri* B | 9·25 | 8·48 | 9·30 | 9·02 |
| *Lact. paracasei* S | 9·34 | 7·58 | 7·50 | 3·70 |
| *Lactobacillus* PRO 244 | 7·91 | 6·38 | ND | ND |

ND, not detected.

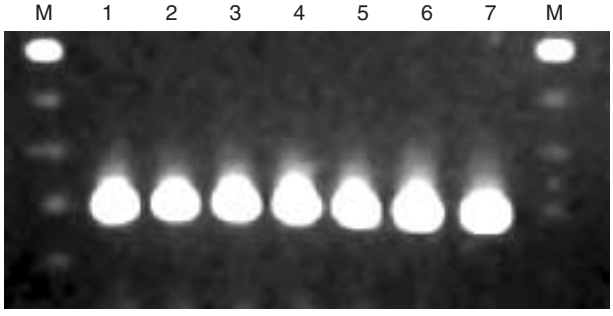


**Fig. 1** PCR amplification of partial gene encoding glycerol dehydratase. Lanes 1–6: PRO 108, PRO 109, PRO 133, PRO 137, PRO 149, PRO 152 respectively. Lane 7: *Lactobacillus reuteri* CECT 925T. Lanes M: 100 bp ladder DNA marker

Five of the six selected *Lact. reuteri* isolates from pig faeces examined in the present work exhibited BSH activity, whereas the reference strain and the commercial strains were negative. Diameters of precipitation zones were measured after spotting cultures on BSH screening medium, with values ranging from 12 to 20 mm for positive isolates. BSH activity is considered a factor involved in the capacity of bacteria to colonize the intestinal tract. The enzyme catalyses the hydrolysis of glycine or taurine-conjugated bile acids into the amino acid residue and the bile acid. Deconjugation through BSH is considered to be a detoxification mechanism to protect bacteria from the toxicity of conjugated bile acids (De Smet *et al.* 1995). However, experimental results have shown that resistance to toxicity and capacity to express BSH activity appear to be independent properties. *Lactobacillus paracasei* and *Lact. casei* showing significant growth in the presence of physiologically high bile salt concentration (10 mmol $l^{-1}$) did not hydrolyse glycocholic and taurocholic acids (TCA) (Haller *et al.* 2001). A total of 26 *Lactobacillus* strains expressed taurodeoxycholic acid (TDCA) and TCA hydrolase activity but only 15 were resistant to TDCA (Moser and Savage 2001). Of 297 strains of *Lactobacillus* spp. from pig faeces screened for BSH activity on plates, 191 were positive (Du Toit *et al.* 1998).

Another important characteristic for strains of lactobacilli to be effective probiotics is the production of bacteriocins antagonistic to pathogens. Neutralized supernatants from cultures grown in MRS broth were tested by an agar diffusion assay against a panel of indicator strains to investigate the presence of bacteriocin-like compounds. Inhibitory activity shown by our isolates was poor, mostly restricted to *C. tyrobutyricum* which was inhibited by the six isolates tested, whereas no inhibitory activity was observed for the reference strain and the commercial strains tested. Supernatants from *Lact. reuteri* PRO 108 and PRO 133 presented antilisterial activity. No inhibitory activity was observed against *Ent. faecalis, Lact. buchneri, Lact. plantarum* or *E. coli*. Research is in progress to determine the nature of the antimicrobial activities responsible for the inhibition. Reutericyclin, a novel tetramic acid derivative produced by *Lact. reuteri* LTH2584 (Gänzle *et al.* 2000) exhibits a broad antimicrobial spectrum. The narrow spectrum detected in the present work points to the presence of bacteriocin-like substances.

In conclusion, a high proportion of lactobacilli isolated from pig faeces have shown the ability to produce reuterin in the presence of glycerol. The levels of reuterin produced by 28 new *Lact. reuteri* isolates were considerably higher than that of type strain *Lact. reuteri* CECT 925T. Six selected isolates, identified as *Lact. reuteri*, were able to survive at conditions likely to be encountered throughout the gastric passage and the small intestine. Five of them presented BSH activity, and further characterization suggested their potential as probiotic bacteria.

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