

## Evaluation of Reuterin Production in Urogenital Probiotic *Lactobacillus reuteri* RC-14<sup>∇</sup>

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**Classified as a distinct species in 1980, *Lactobacillus reuteri* strains have been used in probiotic formulations for intestinal and urogenital applications. In the former, the primary mechanism of action of *L. reuteri* SD2112 (ATCC 55730) has been purported to be its ability to produce the antibiotic 3-hydroxypropionaldehyde (3-HPA), also known as reuterin. In the vagina, it has been postulated that probiotic *Lactobacillus reuteri* RC-14 does not require reuterin production but mediates a restoration of the normal microbiota via hydrogen peroxide, biosurfactant, lactic acid production, and immune modulation. The aim of the present study was to determine whether strain RC-14 produced reuterin. Using PCR and DNA dot blot analyses, numerous *Lactobacillus* species, including RC-14, were screened for the presence of the gene encoding the large subunit of glycerol dehydratase (*gldC*), the enzyme responsible for reuterin production. In addition, lactobacilli were grown in glycerol-based media and both high-performance liquid chromatography and a colorimetric assay were used to test for the presence of reuterin. *L. reuteri* RC-14 was determined to be negative for *gldC* sequences, as well as for the production of reuterin when cultured in the presence of glycerol. These findings support that the probiotic effects of *L. reuteri* RC-14, repeatedly demonstrated during numerous studies of the intestine and vagina, are independent of reuterin production.**

In 1980, Kandler et al. (16) reclassified *Lactobacillus fermentum* biotype II into *Lactobacillus reuteri* species, named after Gerhard Reuter, a German microbiologist who had worked on these organisms. Multiple studies have since shown *L. reuteri* to be a fairly universal species to the intestinal tracts of not only humans (30) but numerous animal species (23, 24, 32), and it is frequently isolated from natural environments as well as many meat and dairy products (31, 37). Based largely upon this ubiquitous and dominant nature, especially among healthy individuals and animals, work began to investigate whether strains of *L. reuteri* could be used to modulate intestinal health. In 1986, a yogurt and milk fermented with a strain of *L. reuteri* reduced the intestinal coliform count in pigs (26), and it was later proposed by Talarico et al. (35) that the beneficial effects were due to the production of reuterin, more specifically 3-hydroxypropionaldehyde (3-HPA), a by-product of glycerol fermentation (9). A follow-up study characterized reuterin as a broad-spectrum antibiotic, effective against both gram-positive and -negative strains of bacteria, as well as several fungi and protozoa (36). Subsequent to this, *L. reuteri* strains have been used commercially as probiotic agents (“live microorganisms which when administered in adequate amounts confer a health benefit on the host”) (11), with the presumption that they produce reuterin.

However, it has become apparent that this antibiotic com-

pound is not unique to *L. reuteri* species. Rather, it has been shown to be produced by potentially pathogenic *Enterobacter agglomerans*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Aerobacter aerogenes* (3, 25, 34), as well as by an intestinal constituent, *Clostridium butyricum* (13). The *L. reuteri* are not the only *Lactobacillus* species to produce reuterin, with an *L. coryniformis* cheese isolate (21), as well as *L. collinoides* (33), also found to be a producer.

In addition, it has recently been shown that not all *L. reuteri* strains produce this antibiotic (22). This raises interesting questions. Just how common is reuterin production among the microbial world? Should non-reuterin-producing *L. reuteri* be given a different designation than those that produce the compound? Are there other important differences among *L. reuteri* strains that would support this reclassification?

In 1998, *L. acidophilus* RC-14, a probiotic isolated in 1985 and described for its anti-infective properties in 1987 (28), was reclassified as *L. fermentum* RC-14 based on ribotyping analysis (41). In 2006, the strain was tested by DNA-DNA hybridization and reclassified as *L. reuteri* RC-14. By then, it had been used in numerous clinical studies and shown to populate the vagina and reduce the risk of infection (2, 27, 29). In vitro studies led to the theory that this strain acted through its ability to produce biosurfactants, hydrogen peroxide, and lactic acid and to modulate the immune response (14, 19, 20). In order to rule out any involvement of reuterin in the activity of the strain, which would make this the first non-reuterin-producing *L. reuteri* probiotic strain, the following methods were used: testing for the presence of the gene encoding the large subunit of glycerol dehydratase (*Gld*), the enzyme required to produce 3-hydroxypropionaldehyde from glycerol (9), and the use of

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both high-performance liquid chromatography (HPLC) and a colorimetric assay to detect reuterin directly within culture supernatants after growth in glycerol.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The following strains were utilized in the study: *L. reuteri* SD2112 (ATCC 55730), *L. reuteri* 1063, *L. reuteri* 1068, *L. reuteri* ATCC 23272T (T-type strain), *L. reuteri* RC-14, *L. reuteri* 359, *L. reuteri* 656, *L. amylovorus* 20552, *L. casei* 393T, *L. crispatus* 33820, *L. delbrueckii* 20074T, *L. fermentum* 14 (Fuller strain) (4), *L. fermentum* 11739, *L. gasseri* 33323, *L. johnsonii* 20553, *L. plantarum* 14917T, *L. rhamnosus* GR-1, *L. rhamnosus* 7469T, and *Escherichia coli* C1214. For genomic DNA isolation, lactobacilli were cultured using MRS media (Becton Dickinson [BD], Oakville, Canada) for 48 h in anaerobic jars containing CO<sub>2</sub>-generating gas packs (BD). *E. coli* was cultured aerobically using brain-heart infusion media for 24 h with shaking (200 rpm). The growth conditions utilized for reuterin production and detection are described in their appropriate sections.

**DNA isolation and PCR screening.** Genomic DNA (gDNA) was isolated from each bacterial strain by using InstaGene matrix (Bio-Rad, Mississauga, Ontario, Canada) according to the manufacturer's supplied protocol. Briefly, one to two bacterial colonies were isolated from each strain, washed once with sterile water, resuspended in 200  $\mu$ l of InstaGene matrix, and incubated at 56°C for 30 min. Each suspension was then mixed, incubated in a boiling water bath for 8 min, mixed again, and centrifuged at 14,000  $\times$  g for 3 min. The resulting supernatant containing gDNA was removed and stored at -20°C until use.

All PCR primers and reagents were purchased from Invitrogen (Burlington, Ontario, Canada), and the gel electrophoresis reagents and equipment were from Bio-Rad. To amplify a portion of the gene encoding the large subunit of Gld (*gldC*), degenerate oligonucleotide primers were generated based upon regions of the gene highly conserved across currently published *Lactobacillus gldC* sequences (*L. hilgardii* [GenBank accession no. AY061969], *L. diolovorans* [AY061968], *L. collinoides* [AY061967], and *L. reuteri* 23272T [also termed F275, CP000705]). The primer sequences generated were: GDCRev-5'-GC[A/G]GC[C/T]TT[G/C]ATATCT[G/T][G/C]AACCAT-3' (matching nucleotides 1800525 to 1800547 from CP000705) and GDCFor-5'-GC[A/C]TA[C/T]GC[A/T]GAAA CCATTCAGTTTA-3' (matching nucleotides 1801252 to 1801227). As a positive DNA control, a portion of the 16S rRNA gene was also amplified from all samples by using the eubacterial primers 16SFor-5'-ACTCCTACGGGAGGCA GCAG-3' and 16SRev-5'-GTATTACCGCGCTGCTGGCAC-3', highly conserved among bacteria (40). Each 50- $\mu$ l PCR contained 100 pmol of each of the corresponding forward and reverse primers, 1 $\times$  PCR buffer, 2 mM MgCl<sub>2</sub>, 200  $\mu$ M deoxynucleoside triphosphates, 5  $\mu$ l (~0.5  $\mu$ g) of gDNA, and 2.5 U of *Taq* polymerase. A total of 30 cycles of amplification were performed consisting of 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C. A single initiation step of 5 min at 94°C prior to cycling and a final elongation step of 10 min at 72°C were also performed to optimize the accuracy and quantity of product produced. Products were separated on 1.2% agarose gels, stained with ethidium bromide, and visualized by using the GelDoc XR system. The expected product sizes were 728 bp for *gldC* and 201 bp for the 16S rRNA gene.

**DNA probe generation and dot blot analysis.** A fragment of the expected 728-bp size was amplified from *L. reuteri* SD2112 gDNA by using GDC primers. This fragment was purified by using a PCR product purification spin column (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's supplied protocol, sequenced (Robarts Research Institute Sequencing Facility, London, Ontario, Canada), and used as a template in a PCR to generate a digoxigenin (DIG)-labeled probe. PCR was performed as already described except that the standard 200  $\mu$ M dTTP used was reduced to 133  $\mu$ M, and 67  $\mu$ M DIG-dUTP added (Roche, Mississauga, Ontario, Canada). The probe was purified via spin column as described above, eluted in 50  $\mu$ l of double-distilled H<sub>2</sub>O, and stored at -80°C until use. In a similar fashion, a 201-bp DIG-labeled probe corresponding to the 16S rRNA gene was generated from *E. coli* C1214 gDNA.

Two identical DNA dot blots were created by applying 2.5  $\mu$ g of gDNA from selected bacterial strains in 15  $\mu$ l of dilution buffer (10 mM Tris-HCl [pH 8.0], 50  $\mu$ g of herring sperm DNA/ml) to positively charged nylon membranes (Roche) using a dot blot manifold (Schleicher and Schuell, Keene, NH) under vacuum. DNA was fixed to the membranes by a 1-min exposure to UV light on a transilluminator, and the blots were air dried. The blots were hybridized and detected in parallel (*gldC* and 16S rRNA gene) using the protocols and reagents outlined in Roche's DIG application manual for filter hybridization. Briefly, the blots were prehybridized for 30 min at 42°C in 10 ml of DIG-Easy Hyb solution in 15-ml conical tubes using a rotating hybridization oven (BioCan, Mississauga,

Ontario, Canada). Fifteen microliters (~250 ng) of each generated probe was separately added to 85  $\mu$ l of double-distilled H<sub>2</sub>O and boiled for 5 min in a boiling water bath to denature the probes into single strands. The probes were then chilled for 2 min on ice and added to 5 ml of prewarmed (42°C) DIG-Easy Hyb. The prehybridization solution was poured off and replaced with the probe solution, and the blots were incubated for 10 h at 42°C with constant rotation. The probes were poured off, and the blots were incubated twice for 15 min each at room temperature in a low-stringency buffer (2 $\times$  SSC [1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate), followed by three 10-min washes in a high-stringency buffer (0.5 $\times$  SSC, 0.1% sodium dodecyl sulfate) at 65°C to remove nonspecifically bound probe. The blots were blocked for 30 min in DIG blocking solution (1% blocking reagent in 0.1 M maleic acid-0.15 M NaCl [pH 7.5]) and incubated with anti-DIG alkaline phosphatase antibody (75 mU/ml in blocking solution) for 30 min. Excess antibody was removed by two 15-min washes in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20) at room temperature, and the blots were equilibrated for 2 min in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl [pH 9.5]). Colorimetric detection was performed in 340  $\mu$ g of NBT (4-nitroblue tetrazolium chloride)/ml and 175  $\mu$ g of BCIP (5-bromo-4-chloro-3-indolylphosphate)/ml in detection buffer until the desired color achieved. NBT and BCIP react with the alkaline phosphatase conjugated to the anti-DIG antibody, resulting in the formation of blue dye, which precipitates on to the membrane. The blots were scanned and stored in TE buffer (pH 8.0) to stop the colorimetric reaction.

**HPLC analysis.** *L. reuteri* 1063 and *L. reuteri* 1068, *L. reuteri* SD2112, and *L. reuteri* RC-14 were cultured and harvested by the homologous method as described Dobrogosz and Lindgren (8). In short, the strains were inoculated in 1 liter of LCM medium (Trypticase, 10 g/liter; yeast extract, 5 g/liter; tryptose, 3 g/liter; KH<sub>2</sub>PO<sub>4</sub>, 3 g/liter; ammonium citrate, 1.5 g/liter; sodium acetate [trihydrate], 1 g/liter; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.2 g/liter; MnSO<sub>4</sub> · H<sub>2</sub>O, 0.13 g/liter; FeSO<sub>4</sub> · 7H<sub>2</sub>O, 60 mg/liter; cysteine-HCl, 0.2 g/liter; Tween 80, 1 ml/liter). The pH was adjusted to 7.0. Sterile glucose was added to 20 mM after sterilization, followed by incubation for 48 h at 37°C. The cells were harvested by centrifugation and washed twice in sterile sodium phosphate buffer (pH 7.5). The cells were then suspended in 10 ml of sterile 250 mM glycerol and incubated for 6 h at 37°C. The suspension was centrifuged, and the supernatant was recovered, sterile filtered through a 0.45- $\mu$ m-pore-size filter, and stored aseptically at 2°C until HPLC analysis. HPLC was performed as described Dobrogosz and Lindgren (8), except that a Shodex Sugar SH1011 (Phenomenex) column was used. The substance eluting at the expected elution time for reuterin was not further analyzed.

**Colorimetric assay for reuterin detection.** The detection of reuterin was performed by using the method of Circle et al. (6). Overnight *L. reuteri* cultures were centrifuged at 4,000 rpm for 10 min at room temperature to pellet the cells. The cells were then washed twice with 50 mM potassium phosphate buffer (pH 7.5), and 100-mg portions were resuspended in 14 ml of 250 mM glycerol, followed by incubation at 37°C for 1 to 2 h. After the cells were pelleted as described above, the supernatant was passed through a 0.45- $\mu$ m-pore-size syringe filter and stored at 4°C. A total of 300  $\mu$ l of each supernatant was added to 225  $\mu$ l of the 10 mM tryptophan solution, 900  $\mu$ l of concentrated HCl was added, and the solution was incubated for 20 min at 37°C. The absorbance was measured at 560 nm by using a SpectraMax M5 microplate reader.

## RESULTS

**GldC gene screening.** The conversion of glycerol to reuterin is known to be dependent upon the activity of Gld, an enzyme that dehydrates glycerol into the aldehyde through the removal of one molecule of H<sub>2</sub>O. Thus, we initially wanted to determine whether *L. reuteri* RC-14 possessed at least one of the genes necessary for its expression. Since *gldC* encodes the largest subunit of the three subunit enzyme (*gldD* and *gldE* encode the other two), we selected it for screening to provide the largest sequence in which to search for highly homologous regions to use in designing oligonucleotide primers and a DNA probe. Based upon the alignment of published *gldC* sequences taken from four species of *Lactobacillus* (*L. hilgardii*, *L. coryniformis*, *L. diolovorans*, and *L. reuteri*), degenerate primers were synthesized. Primers GDCRev and GDCFor were 23 nucleotides in length with five sites of degeneracy and 26 nucleotides

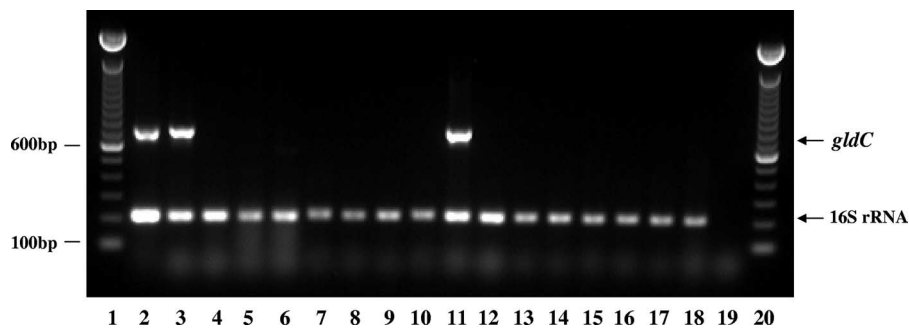


FIG. 1. PCR screening of *Lactobacillus* strains for *gldC* sequence. Upper band at 728 bp represents the expected product using the *gldC*-specific primers, while the lower band at 201 bp represents that expected using the 16S rRNA gene primers (positive DNA control). Lanes: 1 and 20, 100-bp ladder; 2 to 6, *L. reuteri* strains 23272T (positive strain control), SD2112, RC-14, 359, and 656, respectively; 7, *L. amylovorus* 20552; 8, *L. casei* 393T; 9, *L. crispatus* 33820; 10, *L. delbrueckii* 20074T; 11, *L. fermentum* 14; 12, *L. fermentum* 11739; 13, *L. gasseri* 33323; 14, *L. johnsonii* 20553; 15, *L. plantarum* 14917T; 16, *L. rhamnosus* GR-1; 17, *L. rhamnosus* 7469T; 18, *E. coli* C1214 (negative strain control); 19, negative DNA control.

long with three degenerate bases, respectively, and represented 100% matches for all four *Lactobacillus* sequences. PCR screening was performed on 16 *Lactobacillus* strains comprising 10 different species and included both positive (*L. reuteri* 23272T) and negative (*E. coli* C1214) control strains (Fig. 1). Three strains were positive for the expected 728-bp product, namely, *L. reuteri* 23272T, *L. reuteri* SD2112, and *L. fermentum* 14. Strain *L. reuteri* RC-14, as well as the 12 other lactobacilli tested, including two *L. reuteri* clinical isolates, were negative for *gldC* using this method. Based upon the  $T_m$  of the primer sequences and preliminary experiments involving the positive and negative control strains, an annealing temperature of 55°C was used for all PCRs. However, to ensure that the selected annealing temperature was not too high for the degenerate primers to cause false negatives in the reaction, identical PCRs were also carried out with an annealing temperature of 50°C. Similar results were obtained in terms of the amplification of the 728-bp *gldC* product (data not shown).

To confirm the PCR screening results, we generated two identical dot blots containing gDNA from three lactobacilli, namely, *L. reuteri* SD2112 (PCR positive), *L. fermentum* 14

(PCR positive), and *L. reuteri* RC-14 (PCR negative), as well as several controls. Blots were hybridized in parallel with DIG-labeled 728-bp *gldC*-specific and 201-bp 16S rRNA gene-specific DNA probes, respectively (Fig. 2). Probes were generated by PCR similarly to PCR screening but with the addition of DIG-labeled dUTP to the nucleotide mix and were evaluated via gel electrophoresis, showing a single band for each migrating ~1.3-fold greater in size from their corresponding unlabeled products due to DIG incorporation (data not shown). The blotting results for the three lactobacilli were shown to be identical to those observed via PCR, supporting that *L. reuteri* RC-14 does not possess *gldC*.

**HPLC and colorimetric assay.** To augment the genetic screening results, *L. reuteri* RC-14 was cultured in glycerol, known to stimulate reuterin production in other *L. reuteri* strains, to examine whether or not it could produce the compound under these conditions. The controls *L. reuteri* 1063 and *L. reuteri* SD2112 are known to produce reuterin (18, 39), while *L. reuteri* 1068 is known not to produce it with glycerol in the medium. After a 6-h glycerol incubation, culture supernatants from all four strains were examined via HPLC (Fig. 3). As

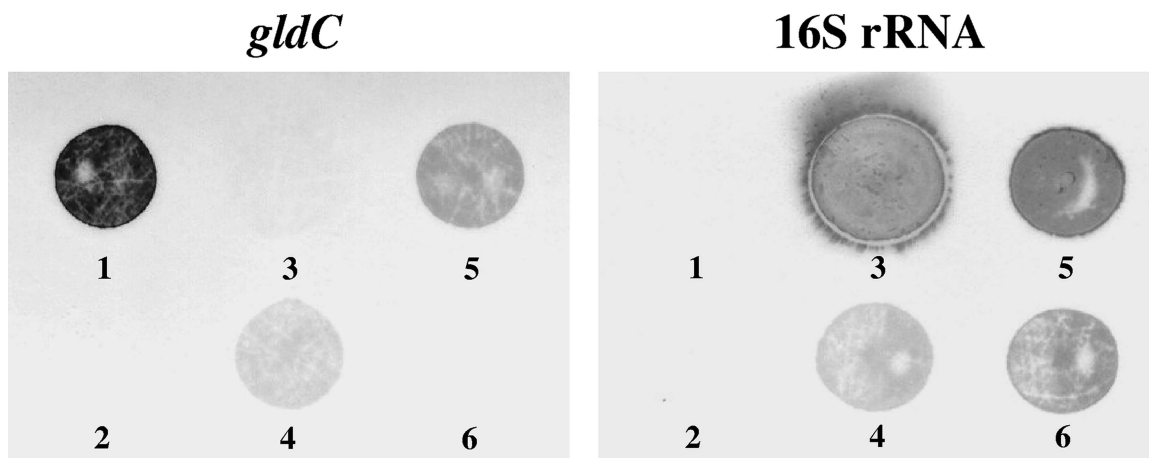


FIG. 2. DNA hybridization screening for *gldC*. Two identical DNA dot blots were prepared and separately screened for *gldC* (left) and 16S rRNA (right) gene sequences using DIG-labeled DNA probes. Spot 1, 5 ng of *gldC* PCR product (positive control); spot 2, DNA dilution buffer only (negative probe control). Spots 3 to 6 each contained 2.5 µg of gDNA from the following strains: spot 3, *E. coli* C1214 (negative strain control); spot 4, *L. fermentum* 14; spot 5, *L. reuteri* SD2112; and spot 6, *L. reuteri* RC-14.

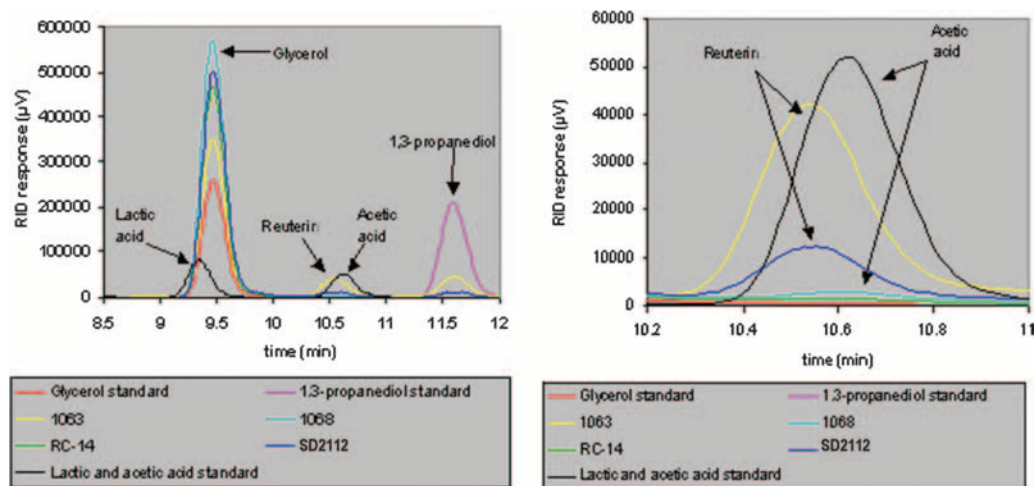


FIG. 3. HPLC analysis of culture supernatants collected from *L. reuteri* strains. The left panel shows the time interval from 8.5 to 12 min of chromatograms of the four samples and standard compounds. The right panel shows an enlarged section of the chromatogram from 10.2 to 11 min. The data clearly demonstrate that there is a real-time difference between the reuterin peak (10.55 min) eluting 6/100 min before acetic acid (10.61 min) and the absence of reuterin in strain *L. reuteri* RC-14. Glycerol was the test substrate, and lactic and acetic acids are normal by-products of *Lactobacillus* glycerol fermentation. 1,3-Propanediol is a compound produced via the reduction of reuterin and therefore should not be present in the supernatants of non-reuterin-producing strains.

expected, both *L. reuteri* 1063 and *L. reuteri* SD2112 showed a peak at the expected elution time for reuterin between glycerol and 1,3-propanediol, whereas *L. reuteri* 1068 and *L. reuteri* RC-14 did not. Thus, RC-14 cannot be induced to produce reuterin in the presence of glycerol, conditions known to stimulate its production in other *L. reuteri* strains.

To support the HPLC results, we also monitored the production of reuterin by using a colorimetric assay. Two independent isolates of *L. reuteri* RC-14 were grown to stationary phase in MRS medium at 37°C. Cells were pelleted, washed once with phosphate-buffered saline, and resuspended in 15 ml of 250 mM glycerol. As a positive control, a reuterin-producing *L. reuteri* strain (ATCC 6475) was used. As a negative control, we utilized a *L. reuteri* strain in which the *gldC* gene was disrupted. As expected, only the positive control *L. reuteri* strain produced any colorimetric change, indicating the presence of reuterin (optical density at 560 nm [OD<sub>560</sub>] = 0.628). The two independent isolates of *L. reuteri* RC-14 (OD<sub>560</sub> = 0.001, 0.000) and the *gldC* mutant (OD<sub>560</sub> = 0.001) did not yield any colorimetric change, indicating a lack of production of reuterin.

## DISCUSSION

This study has shown that probiotic *L. reuteri* RC-14 does not appear to contain a *gld* gene, and reuterin production was not detected in this strain. This is important for a number of reasons. First, it indicates that reuterin production is not a mechanism of action of *L. reuteri* RC-14 to confer health benefits on the intestine and vagina, as this strain has been shown to do (1, 27). Thus, the long-held view that specific antimicrobial substances are required for probiotic strains to function and to displace pathogens is not supported by the present findings. Rather, probiotic strains such as *L. reuteri* RC-14 can function in other ways that are antagonistic to pathogen colonization, including modulating host immunity (17, 20) and

producing biosurfactants and other antiadhesive factors (19, 38). This emphasizes that selection of a strain intended for probiotic use cannot be solely made based upon the presence of an antimicrobial compound. This supports the position of the Food and Agriculture Organization of the United Nations and World Health Organization, whose report (12) states this very point. However, the production of antibiotic-type substances is still being used as a means to identify and propagate new probiotic strains (7, 10).

Second, it reemphasizes the diversity of probiotic strains, even within the same species, and shows that species possess different functional properties. Such phenotypic differences between strains have been seen previously, for example, with *L. rhamnosus* GR-1 better functioning in the vagina than *L. rhamnosus* GG (5), which is better known for its intestinal modalities (15).

In the case of *L. reuteri* RC-14 and similar strains that do not produce reuterin, several questions arise. Should they become members of a nonreuterin subspecies of *L. reuteri* and studied further to determine whether additional characteristics warrant their designation as a novel species of *Lactobacillus*? Or, given that reuterin is produced by strains other than *L. reuteri*, should this compound be renamed? The reclassification of bacterial species is generally based on genotypic profiles, even though these, too, can differ between isolates. However, since strain *L. reuteri* RC-14 is also genetically closely related to *L. fermentum*, as shown by ribotyping (41), and does not possess the genes required to produce a factor key to the identification and designation of *L. reuteri* strains, should it remain under this classification?

The factors that influence the evolution of bacterial species evolve with new techniques. However, the strain itself remains the same. Thus, *L. reuteri* RC-14 was formerly named *L. acidophilus* RC-14 (28) and *L. fermentum* RC-14 (27), and its probiotic properties predate the discovery of reuterin-produc-

ing *L. reuteri* strains. Since the naming and speciation of strains has more and more recently had commercial implications with probiotic products appearing in various forms around the world, it behooves microbiologists to carefully outline the exact criteria for strain speciation, beyond simple genomic profiles.

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