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BIOCONVERSION OF CRUDE GLYCEROL INTO REUTERIN BY LACTOBACILLI ISOLATED FROM SILAGE

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ABSTRACT

Heterofermentative lactobacilli were isolated from sorghum and maize silage, and their ability to produce the antimicrobial compound reuterin (3-hydroxypropionaldehyde, 3-HPA) was investigated. Additionally, reuterin production from crude and partially purified biodiesel-derived glycerol was evaluated as an alternative to the utilization of these by-products. Only two out of 148 lactobacilli isolated produced reuterin in the presence of glycerol. The gene encoding a subunit of glycerol dehydratase was detected by PCR in isolates SO8 and SO23, identified as *Lactobacillus reuteri* by 16S rRNA gene sequence analysis. Levels of reuterin produced by these two isolates and strain *L. reuteri* DSM 17938, used as control, were different. For each bacteria, the amount of reuterin detected from biodiesel-derived glycerol was comparable with that obtained from aqueous solution of pure glycerol. Among silage isolates, *L. reuteri* SO23 yielded the highest levels of reuterin production. In addition, cell-free supernatants revealed inhibitory activity against silage spoilage microorganisms like spore-forming bacteria, yeast and mould. *L. reuteri* SO23 proved to be a valuable candidate for use of crude glycerol and its bioconversion to reuterin. Addition of glycerol together with reuterin-producing lactobacilli strains could represent a good alternative as silage biopreservant and help optimize profit in biodiesel production.

Keywords: *Lactobacillus reuteri*, reuterin, silage, biodiesel-derived glycerol

INTRODUCTION

Glycerol has become an abundant resource as by-product of the biodiesel industry (**Stelmachowski, 2011**). The utilization of waste glycerol for fine chemical synthesis could reduce pollution and bring down the biodiesel production costs. A wide variety of microorganisms can utilize glycerol as substrate for the production of cell biomass and high value-added compounds through oxidative and reductive pathways. Microbial bioconversion of glycerol results in the synthesis of a variety of chemicals like 1,3-propanediol (1,3-PDO), dihydroxyacetone, citric acid, glyceric acid, 3-hydroxypropionaldehyde, succinic acid, erythritol, polyhydroxyalkanoates and other compounds (**Dobson *et al.*, 2012**). Some lactic acid bacteria (LAB) belonging to the genus *Lactobacillus* produce the antimicrobial compound reuterin (3-hydroxypropionaldehyde or 3-HPA) during anaerobic metabolism of glycerol (**Talarico and Dobrogosz, 1989; Vollenweider *et al.*, 2010**). Bioconversion of crude glycerol by LAB into a compound with antimicrobial activity could lead to new biothecnological application for this waste product, and the development of new bioprotective microbial cultures.

The search for bioprotective cultures is a topic in development in many areas of microbiology, including silage microbiology. Silage is feedstuff produced by the fermentation of crop or forage under anaerobic conditions. As a result of water-soluble carbohydrate consumption mainly by LAB, lactic acid is produced and pH below 4 is achieved. One of the main problems that affect the nutritional quality and safety of silage is aerobic deterioration. Yeasts that degrade lactic acid in the presence of air trigger aerobic spoilage. In combination with mould development, silage becomes unstable and heats rapidly. Degradation of lactic acid increases pH values to levels that allow the development of less acid tolerant microorganisms, like spore-forming bacteria. Aerobic (*Bacillus* spp.) and anaerobic (*Clostridium* spp.) spore-forming bacteria increase the impact of spoilage by hydrolytic enzyme production and undesired fermentation. Aerobic spoilage is the major cause of reduced nutritional value of silage and increases the risk of development of potential pathogenic microorganisms frequently associated with silage like enterobacteria, *Listeria* spp. and *Salmonella* (**Queiroz *et al.*, 2018**). In addition, moulds that develop in air-exposed silages may produce mycotoxins, which are detrimental to the health of humans and animals (**Pahlow *et al.*, 2003**). Lactic acid bacteria, mainly *Lactobacillus* spp., are the most common biological additives used to preserve silage worldwide. Main effects of bacterial inoculants are preservation by acidification during storage, and prevention of aerobic spoilage of opened silos. A highly desirable attribute of silage bacterial inoculants, besides lactic acid production, is production of antimicrobial substances (**Duniére *et al.*, 2013**).

Some LAB belonging to the genus *Lactobacillus* produce a non-protein antimicrobial compound, soluble in water, resistant to heat and stable over a wide pH range (2-8), known as reuterin (3-hydroxypropionaldehyde or 3-HPA) (**Talarico *et al.*, 1988**; **El-Ziney and Debevere, 1998**). Reuterin is produced during anaerobic metabolism of glycerol as an intermediate step in the conversion of glycerol to 1,3-propanediol (**Lüthi-Peng *et al.*, 2002**). Enzymes participating in this bioconversion pathway are under the control of the propanodiol (*pdu*) utilization operon (**Morita *et al.*, 2008**). This low molecular weight compound is active against a wide range of Gram-positive bacteria such as *Staphylococcus*, *Listeria*, *Clostridium* and *Bacillus*, as well as Gram-negative like *Escherichia coli*, *Yersinia*, *Shigella*, *Pseudomonas* and *Salmonella* (**Arqués *et al.*, 2004**; **Axelsson *et al.*, 1989**; **Bian *et al.*, 2011**; **Cleusix *et al.*, 2007; El-Ziney and Debevere, 1998**; **Spinler *et al.*, 2008**). Reuterin also inhibits growth of several moulds and yeasts, including *Candida albicans*, *Pichia* sp., *Kluyveromyces* sp. (**Tanaka *et al.*, 2009**; **Martin *et al.*, 2005**), *Aspergillus flavus* (**Axelsson *et al.*, 1989**) and *Penicillium* sp. (**Martin *et al.*, 2005**). Some strains of *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus collinoides*, *Lactobacillus coryniformis* and *Lactobacillus reuteri*, excrete reuterin under anaerobic conditions (**Schütz and Radler, 1984; Claisse and Lonvaud-Funel, 2000**; **Nakanishi *et al.*, 2002**; **Gómez-Torres *et al.*, 2014**). These reuterin-producing *Lactobacillus* strains could be considered as potential bioprotective cultures, and some examples have been described. The application of these bacteria with glycerol generated a suitable environment for *in situ* production of reuterin, reaching adequate concentrations to control pathogens that may be present during the manufacture and storage of dairy products (**Langa *et al.*, 2013**). In the same way, inoculation of *L. coryniformis* plus glycerol inhibited butyric fermentation and retarded yeast and mould growth, preventing rice straw ensiling spoilage (**Tanaka *et al.*, 2009**).

Reuterin-producing *Lactobacillus* could be potential silage inoculants to control spoilage microorganisms and prevent butyric fermentation and aerobic spoilage. An additional challenge for this hypothesis is to explore alternative glycerol sources, like biodiesel-derived glycerol, for reuterin production. A modified strain of *Lactobacillus diolivorans* was able to produce high value C3 chemicals

like 3-hydroxypropionialdheyde, 1,3-propanediol and 3-hydroxypropionic acid from crude glycerol (**Lindlbauer *et al.*, 2017**). Other lactobacilli like *L. delbrueckii*, *L. acidophilus*, and *L. plantarum* can efficiently use biodiesel-derived glycerol as carbon source for biomass and energy production, but the reductive pathway involved in the synthesis of reuterin is not expressed in these bacteria (**Rivaldi *et al.*, 2013**). The existence of microbial strains able to produce reuterin from crude glycerol prompted us to analize the potential of silage derived lactobacilli strains to convert biodiesel-derived glycerol into reuterin, with the future perspective of being used as silage inoculant. In this study, heterofermentative lactobacilli isolated from sorghum and maize silage were selected and evaluated for reuterin production using crude and partially purified biodiesel-derived glycerol as substrate.

MATERIALS AND METHODS

Isolation and selection of reuterin-producing lactobacilli

Sorghum (20) and maize (5) bag silage were obtained from different farms located in the South of Uruguay, in areas dedicated to dairy production. Silage samples were homogenized in a laboratory blender Stomacher 400 Circulator (Seward Ltd., Worthing, UK). Decimal dilutions were prepared in 9 mL of 0.1% of sterile peptone water, plated on Man Rogosa Sharpe agar (MRS, Oxoid Ltd, UK) and incubated at 37°C for 48 h under microaerophilic conditions using the Anoxomat MARK II gas exchange system (MART Microbiology B.B., the Netherlands). After incubation, up to five colonies per sample were picked randomly from the plate and purified by streaking on MRS agar. Phenotypic characterization was performed by routine laboratory test: colony morphology, catalase and oxidase test, and Gram staining. Only catalase and oxidase negative Gram-positive rod-shaped isolates were subcultured in MRS broth (Oxoid, UK) at 37ºC for 24 h. Inverted Durham tubes were used to visualize gas production. CO_2 producing isolates were inoculated in 5 mL of MRS broth with glycerol (250 mM) at 37ºC for 24 h. Then, cells were harvested by centrifugation at 10000 rpm for 5 min, suspended in 30 mL of an aqueous solution with 250 mM glycerol and incubated for 3 h at 37ºC under anaerobic conditions. After incubation, cell suspensions were centrifuged at 10000 rpm for 20 min at 4°C and the supernatant was recovered and filtered (0.2 µm pore size; Sartorius Inc., California, USA). Compounds with carbonyl groups, presumptively reuterin, were detected in cell-free supernatants by microplate colorimetric method (see below). Inhibitory activity of cell-free supernatants was determined by disk diffusion assay. Briefly, 100 µL of cell-free supernatant were pipetted onto filter paper disks (diameter 6 mm, Whatman, GE Health Care, NJ, USA) placed on Plate Count Agar (PCA, Oxoid, UK) plates previously inoculated with 100 µL of an overnight culture of *E. coli* DH5α, used as indicator microorganism. This strain was cultured in Tryptic Soy Broth (TSB, Oxoid, UK) (an approximate concentration of 10^4 cells). Clear zones around the disks (> 2 mm) after 24 h of incubation at 37ºC, were considered positives for inhibitory activity (**Martin *et al.*, 2005**).

Determination of antimicrobial spectrum

Disk difussion assay previously described was used to determine inhibitory activity of reuterin-producing isolates against species of spore-forming bacteria, moulds and yeasts. Cell-free supernatants were pipetted onto filter paper disks placed on agar plates previously inoculated with 100 µL of an overnight culture of indicator microorganism. The following microorganisms, from our own culture collection, were used as indicators: *Bacillus licheniformis* UY132, *Bacillus pumilus* UY1030, *Bacillus subtilis* UY1067, *Clostridium tyrobutyricum* UY226.3, *Clostridium sporogenes* UY185.1, *Penicillium* sp. UY12, *Candida* sp. UY22.8 and *Kluyveromyces* sp. UY15.2. As well, inhibitory activity against sorghum silage homogenizate, containing natural occuring moulds and yeasts, was tested. Ten grams of silage were homogenizated with 90 mL of sterilized peptone-water (1,0 g/L peptone and 8,50 g/L NaCl) in a laboratory blender Stomacher 400 Circulator (Seward Ltd., Worthing, UK) for 2 minutes at 260 rpm. Malt Extract Agar plates (MEA, Oxoid, UK) were inoculated with 100 µL of silage homogenizate. Plates with disks, supernatants and bacterial indicators were incubated at 37ºC for 48 h. *Bacillus* spp. were grown in PCA medium and *Clostridium* spp. were grown in Reinforced Clostridium Agar (RCA, Oxoid, UK) under anaerobic conditions. Plates with yeast and mould cells, grown in MEA, were incubated at 27°C for 48 h and 120 h, respectively. Finally, plates were examined for clear zones of inhibition around the disks (> 2 mm). Inhibitory activity experiments were performed in duplicate.

Reuterin production quantification

Reuterin production was quantified by the typical ketone or aldehyde functional group assay described previously by **Tokuyama *et al.* (2014)** for methylglyoxal quantification, with modifications. Reuterin-producer *L. reuteri* DSM 17938 was included as positive control. Isolates were inoculated in 5 mL of MRS broth with glycerol (250 mM) at 37ºC for 24 h. Cells were harvested by centrifugation at 10000 rpm for 5 min, resuspended in 30 mL of an aqueous solution with 250 mM glycerol (Sigma Aldrich, 99.5%) and incubated at 37ºC under anaerobic conditions for 3 h. Cells suspensions were previously adjusted when needed to OD_{600nm}=1 (~ 10^9 CFU/mL). Carbonyl compounds present in filtered cell-free supernatants were determined using a microplate colorimetric assay with 2,4-dinitrophenylhydrazine (2,4-DNPH) (Sigma Aldrich). The reaction mixture containing 200 µL of supernatant and 67 µL of 2,4-DNPH solution (0.1% 2,4-DNPH in 2 M HCl) was incubated for 15 min at 30°C, and then 333 µL of 10% NaOH was added. After 15 min incubation at room temperature, the presence of dinitrophenylhydrazone was determined by absorbance measurements (A_{544nm}). One arbitrary unit (AU) was defined as the amount of dinitrophenylhydrazone required to increase one unit of A_{544nm} under the assay conditions. Reuterin production was determined considering that one mole of dinitropenylhydrazone can be synthesized per mole of reuterin. The assay was also performed by substituting the aqueous glycerol solution for crude glycerol (50 % w/w glycerol) and glycerol partially purified by splitting with HCl (70 % w/w glycerol), adjusted to 250 mM of available glycerol. Crude and partially purified glycerol were obtained as by-product from biodiesel production kindly provided by ALUR Alcoholes del Uruguay (Montevideo, Uruguay). All assays were performed in triplicate and the values were expressed as the mean±S.D. Statistical analysis was performed using analysis of variances ANOVA. Differences were considered statistically significant at $P < 0.05$.

PCR detection of glycerol dehydratase gene

A polymerase chain reaction (PCR) method was conducted to detect the presence of *pduC* gene, encoding a subunit of glycerol dehydratase, the first enzyme in the reuterin formation pathway. Primers pduCF (5′-CCTGAAGTAAAYCGCATCTT-3′), pduCR (5′-GAAACYATTTCAGTTTATGG-3′) and PCR conditions described by **Walter *et al.* (2011)** were used. *L. reuteri* DSM 17938 DNA was included as positive control. Preparation of genomic DNA was performed from bacterial overnight cultures in MRS broth, cells were harvested at 10,000 rpm for 5 min and DNA was purified using a Genomic DNA purification kit (Fermentas International Inc., USA) following the manufacturer's instructions. Purified DNA was suspended in 100 µL TE buffer and used as template in amplification reactions. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific Incorporation, Wilmington, DE, USA).

Identification of selected reuterin-producing isolates

A second PCR reaction was carried out to identify selected isolates at species level. Primers fD1 and rD1 were used to amplify a 1540 bp 16S rRNA gene fragment (**Weisburg *et al.*, 1991**). PCRs were performed using an initial denaturation step at 94 °C for 7 min, then 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min and a final extension at 72°C for 10 min. Amplified fragments were purified and sequenced by Macrogen Sequencing Service, Korea, using an ABI PRISM 3730XL capillary sequencer (Applied Biosystems, CA, USA). To identified the isolates obtained, DNA sequences were compared with the 16S ribosomal RNA sequence database from NCBI (National Center for Biotechnology Information), using the standard Nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

Selection and identification of reuterin-producing isolates

Most bacterial silage inoculants are composed by homofermentative and/or heterofermentative lactobacilli. Few heterofermentative *Lactobacillus* spp. are able to produce reuterin, and this ability is strain specific (**Burgué *et al.*, 2015**). In the present work, from 25 samples of sorghum and maize bag silage, were obtained 148 isolates grown on MRS agar plates. A total of 30 isolates produced gas under anaerobic conditions in MRS broth and were considered as heterofermentative lactobacilli. Isolates were evaluated for their ability to produce carbonyl compounds, presumptively reuterin, under anaerobic conditions by a microplate colorimetric assay. Results are shown in Figure 1A. Positive results were detected in cell-free supernatants of only two isolates (SO8 and SO23). A change of colour, owing to the formation of dinitrophenylhydrazone as an orange or red precipitate was visualized. SO8 and SO23 reuterin-producing isolates were obtained from two different sorghum silage samples. Phenotypic features of these two isolates corresponded to Gram positive, catalase and oxidase negative rods, able to metabolize glucose by the heterofermentative pathway. Most of these characteristics suggested that these isolates could belong to the genus *Lactobacillus*. The occurrence of reuterin-producing lactobacilli in sorghum and maize silage was low (two isolates out of 148). All silage analyzed were commercially inoculated with microbial additives, carriyng several LAB species. Nutrient competitive pressure between inoculated LAB and epiphytic crop bacteria may explain the scarce occurrence. Microplate colorimetric assay resulted in a simple, rapid and effective method that evidence at first glance the presence of reuterin in supernatants and was then used to quantify the concentration of this compound by spectrophotometry. Cell-free supernatants

inhibitory activity on indicator microorganism *E. coli* DH5α was observed by disk difussion assay shown in Figure 1B. The production of an antimicrobial compound, presumed reuterin, by isolates SO8 and SO23 incubated in the presence of aqueous glycerol solution was then evidenced.

Genomic DNA of the two reuterin-producing isolates and control strain were used as templates in PCR reactions designed to amplify part of glycerol dehydratase (*pduC*) gene. A 122 bp amplicon corresponding to a fragment of *pduC* gene, encoding a subunit of glycerol dehydratase, the first enzyme in the propanediol fermentation/reuterin formation pathway, was obtained for isolates SO8 and SO23, as well as for reference strain *L. reuteri* DSM 17938 (data not shown). Molecular screening for reuterin formation pathway enzymes encoding genes is not enough to ensure reuterin production. However, the determination of antimicrobial activity and the detection of ketone or aldehyde functional groups in the supernatant (Figure 1), suggest that isolates SO8 and SO23 could produce reuterin under assayed conditions and the amplified products could be related with its synthesis. To support this, a chemical characterization of the antimicrobial compound using additional analytical techniques, like HPLC should be done (**Tokuyma *et al*., 2014**).

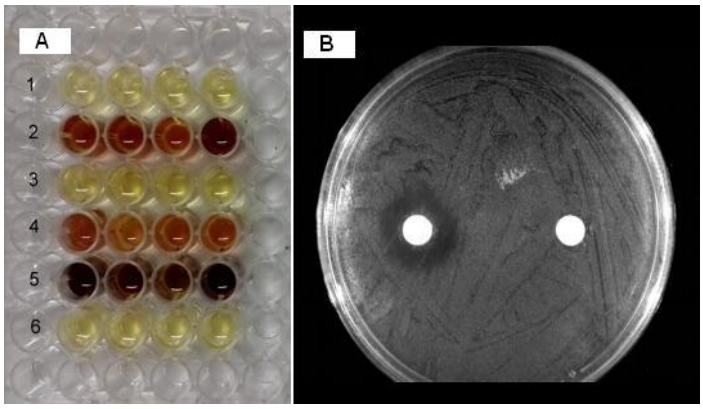

Figure 1 Reuterin-producing screening methodology. A. Colorimetric reaction for reuterin detection in cell-free supernatants in microtiter plates. Lane 5: positive control, lane 6: negative control, lanes 1 and 3: non reuterin-producing isolates, lanes 2 and 4: reuterin-producing isolates. B. Inhibitory activity assay using *E. coli* DH5α as indicator microorganism.

Isolates SO8 and SO23 were identified as *Lactobacillus reuteri* by partial 16S rRNA gene sequence analysis (GenBanK DNA sequence accession numbers: MH487823, MH487824). Several strains of *L. coryniformis* (**Martin *et al*., 2005**), *L. collinoides* (**Sauvageot *et al*., 2000**), *L. buchneri*, *L. brevis* (**Schutz and Radler, 1984**), and *L. reuteri* (**Talarico *et al*., 1988**) are able to produce antimicrobial substance reuterin. Most reuterin-producing strains reported in literature are *L. reuteri* strains. *L. reuteri* is not able to consume glycerol as sole carbon and energy source, because these bacteria don´t express an enzyme with dihydroxyacetone kinase activity, required for channeling the glycerol into central carbon metabolism (**Dishisha *et al*., 2014**).

Reuterin quantification and antimicrobial spectrum

Reuterin concentration was determined in cell-free supernatants by the production of dinitrophenylhydrazone, measured spectrophotometrically. Reuterin production levels by *L. reuteri* SO8 and SO23 are shown in Figure 2. *L. reuteri* DSM 17938 strain was included as positive control. When aqueous glycerol solution was used as substrate, differences in reuterin production were observed among reuterin-producing isolates (P=0,005). DSM 17938 and isolate SO23 exhibited higher values compared to isolate SO8.

The quantification of reuterin included as first step the normalization of cell biomass previous to the incubation with glycerol. Then, the differences observed between isolates should be attributed to differences in the efficiency of glycerol conversion pathway, and not to the number of active cells. Glycerol uptake, enzymatic pathway imbalance and toxic accumulation of 3-hydroxypropionialdheyde (reuterin), could be some of the reasons that explain the differences observed (**Burgué *et al*., 2015, Vollenweider and Lacroix, 2004**). Results indicate that although SO8 and SO23 were identified as *L. reuteri*, they correspond to two strains with different glycerol conversion capabilities.

Antimicrobial spectrum was evaluated using cell-free supernatants of SO8 and SO23 isolates. The supernatants exhibited a broad spectrum of inhibitory action against aerobic and anaerobic spore-forming bacteria (Table 1). Data showed that all tested strains were sensitive to reuterin by the presence of clear zones of inhibition around the disk (> 2 mm). *Bacillus* and *Clostridium* species used for the inhibitory activity assay belongs to our laboratory culture collection and are natural occuring contaminants in dairy farm environment. *Penicillum* sp. and both yeasts tested were also inhibited. Additionally, a silage homogenizate containing natural occuring moulds and yeasts was challenged against reuterin-containing supernatants. The inhibitory effect on this population was evident, limiting the growth of fungi and yeasts colonies around the disk (Figure 3). Sensitivity to reuterin of spore-forming bacteria and the inhibitory activity observed for natural occurring silage contaminants, set up a great expectation for the role of reuterin as silage biopreservant.

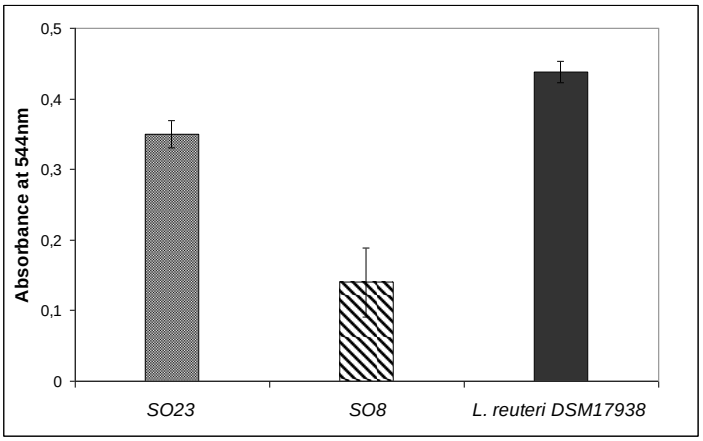

Figure 2 Reuterin production with aqueous glycerol solution as substrate. Dinitrophenylhydrazone concentration was estimated by absorbance measurements at 544nm. Experiments were performed in triplicate, and error bars indicate ±SD.

Table 1 Sensitivity of selected spore-forming bacteria, moulds and yeasts to reuterin.

Species	**Sensitivity***
Bacillus licheniformis	+
Bacillus subtilis	+
Bacillus pumilus	+
Clostridium tyrobutyricum	+
Clostridium sporogenes	+
Penicillium sp.	+
Candida sp.	+
Kluyveromyces sp.	+

* Sensitivity expressed as positive when inhibition halo >2mm.

Reuterin production from biodiesel-derived glycerol

The synthesis of reuterin involves two main steps: the uptake of glycerol into the cell, followed by the intracellular conversion of this substrate into 3-hydroxypropionialdheyde, mediated by the enzyme glycerol dehydratase. The impurities present in crude glycerol can affect the physiology of *L. reuteri*, altering the amounts of glycerol incorporated and reuterin synthesized. The expression of endogenous glycerol uptake facilitating proteins of the microorganism itself can also limit glycerol entrance to the cell (**Lindlbauer *et al*., 2017**). The possibility of using crude glycerol as a substrate depends on the degree of its purity (**Samul *et al*., 2014**). Therefore, to test the feasibility of this work hypothesis, ability of selected strains to effectively produce reuterin from crude and partially purified biodiesel-derived glycerol as a substrate was evaluated. Bioconversion of biodiesel-derived crude glycerol was performed by substituting the aqueous glycerol solution for crude and partially purified biodiesel glycerol. *L. reuteri* DSM 17938 and isolates SO23 y SO8 produced reuterin from crude (50% w/w glycerol) and partially purified (70% w/w glycerol) biodiesel-derived glycerol as substrate (Figure 4). Strain SO8 exhibited the lowest levels of reuterin production under all conditions tested (P<0,05). *L. reuteri* DSM 17938 and SO23 behaved similarly with comparable levels of reuterin production for all substrates (P=0,14). Crude glycerol yielded the lowest reuterin levels for all three strains. The production of reuterin from crude and from partially purified biodiesel-derived glycerol did not exhibit significant differences for isolate SO8 and for the strain DSM 17938 of *L. reuteri* (P>0,05). Levels of dinitrophenylhydrazone detected when partially purified glycerol or aqueous glycerol solution were use for the incubation, were similar for isolate SO23 and for *L. reuteri* DSM17938. Impurities like free fatty acid, inorganic salts, and alcohol are present in higher amount in this substrate and that can limit the efficiency of microbial bioconversion (**Samul *et al*., 2014**). The impurities had a different impact in each organism tested. Available glycerol concentrations ranging from 150 mM to 400 mM are reported as optimal for reuterin production (**Gou *et al*., 2011; Doleyres *et al*., 2005**). The experiments developed in this work were designed adjusting the concentration of available glycerol to 250 mM.

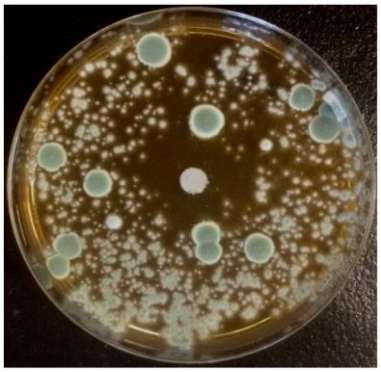

Figure 3 Inhibitory activity of *L. reuteri* SO23 cell-free supernatant against sorghum silage homogenizate containing natural occuring moulds and yeasts.

Like all microbial processes, the bioconversion of glycerol to reuterin is a dynamic process that depends on or can be affected by several factors, like glycerol concentration and purity, pH and temperature. In addition to the genetic background or the intrinsic enzymatic capacity of each *L. reuteri* isolate studied in this work, the quality of glycerol available for the bioconversion process affected the amount of reuterin produced. An important issue is that crude materials, like biodiesel-derived glycerol, have unstandarized composition and diverse quality depending on raw materials and the industrial process. Isolation and selection of microorganisms able to effectively use wastes or industrial by-products is a main issue faced for those interested in introducing such processes into biotechnological applications.

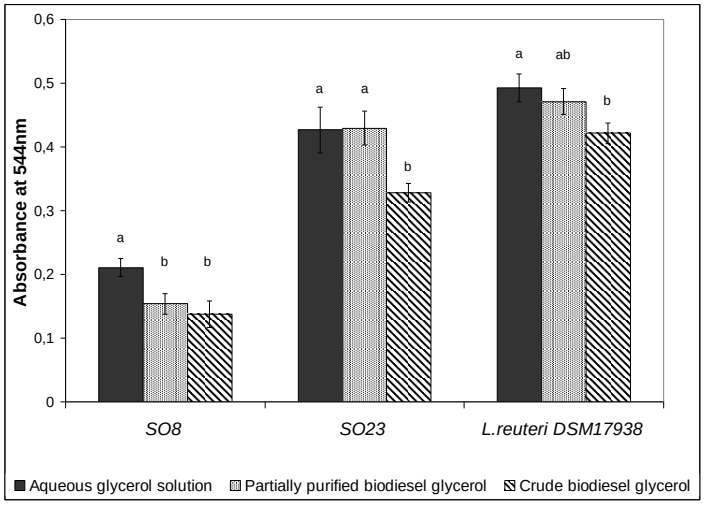

Figure 4 Reuterin production from biodiesel-derived glycerol compared to aqueous glycerol solution. Reuterin content in cell-free supernatants was estimated by the production of dinitrophenylhydrazone (A_{544} nm). Experiments were performed in triplicate, and error bars indicate ±SD. For each isolate, different letters means significant differences between substrates (P<0,05).

CONCLUSIONS

In this work, the production of compounds with carbonyl groups, presumptively reuterin, by two isolates of *L. reuteri* recovered from sorghum silage, is presented. In addition, one of these isolates, *L. reuteri* SO23, produced reuterin from biodiesel-derived glycerol at similar levels to those achieved when it was incubated in the presence of pure glycerol. The inhibitory action of reuterin against most common silage spoilage microorganisms and some of the properties displayed by *L. reuteri* SO23 make it very attractive as a potential silage biopreservant inoculant. More research need to be done for the effective use of these reuterin-producing *L. reuteri* isolates as silage biopreservants, but results observed in this study are encouraging. The approach presented could generate an eco-friendly route for the use of a by-product as biodiesel derived glycerol. Also, this represents an opportunity to integrate crude glycerol, and reuterin-producing *Lactobacillus* spp. in a suitable combination to control silage spoilage microoganisms.

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