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Evolved Probiotics™ *Lr* 3613-1: University of Milwaukee- Wisconsin: Third Party Confirmation of Reuterin Production

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Introduction

Limosilactobacillus reuteri (previously classified as *Lactobacillus reuteri*) strain 3613-1 was identified from a library of over 56,000 lactic acid bacteria as a unique strain for its ability to produce antimicrobial metabolites. *L. reuteri* is naturally found in the intestines and vagina of humans and animals and has the ability to produce lactic acid and Hydrogen peroxide (H_2O_2). The Arm & Hammer™ brand's proprietary strain *L. reuteri* 3613-1 can produce reuterin, a compound that has potent anti-microbial properties. Previous internal studies have validated the production of reuterin from glycerol by *Lr* 3613-1 through HPLC analysis of the bacterial metabolites produced from *Lr* 3613-1. In the present study the University of Wisconsin-Milwaukee (UWM) acted as a third party to validate the ability of *Lr* 3613-1 to metabolize glycerol into reuterin through HPLC analysis.

The objective of this study was to confirm the presence or absence of reuterin and glycerol in bacterial supernatant samples harvested from *Lr* 3613-1 and from the control strain *Lr* RC-14, which is not known to produce reuterin. Cultures were grown in triplicate at the Church & Dwight Co., Inc. Science-Hearted Center, and following incubation, supernatants from each sample were filtered and stored at -20°C until HPLC analysis. Frozen supernatants were subsequently transported to UWM for HPLC quantification of reuterin and glycerol.

Results

Table 1. HPLC Results from UWM.

Sample	Glycerol Concentration (ppm)	Reuterin Concentration (ppm)
Blank	0.000	0.000
Standard 25 ppm	24.27	23.12
Standard 75 ppm	69.30	82.39
Standard 222 ppm	200.16	270.87
Standard 667 ppm	696.43	781.83
Standard 2000 ppm	2,033.68	2,195.69
Standard 6000 ppm	6,020.50	6,545.71
Media Control Replicate 1	24,566.49	0.00
Media Control Replicate 2	25,413.92	0.00
Media Control Replicate 3	27,032.33	0.00
Lr RC-14 Supernatant Replicate 1	22,735.69	0.00
Lr RC-14 Supernatant Replicate 2	23,382.80	0.00
Lr RC-14 Supernatant Replicate 3	24,891.23	0.00
Lr 3613-1 Supernatant Replicate 1	392.40	14,175.80
Lr 3613-1 Supernatant Replicate 2	458.08	14,489.00
Lr 3613-1 Supernatant Replicate 3	569.94	16,213.57

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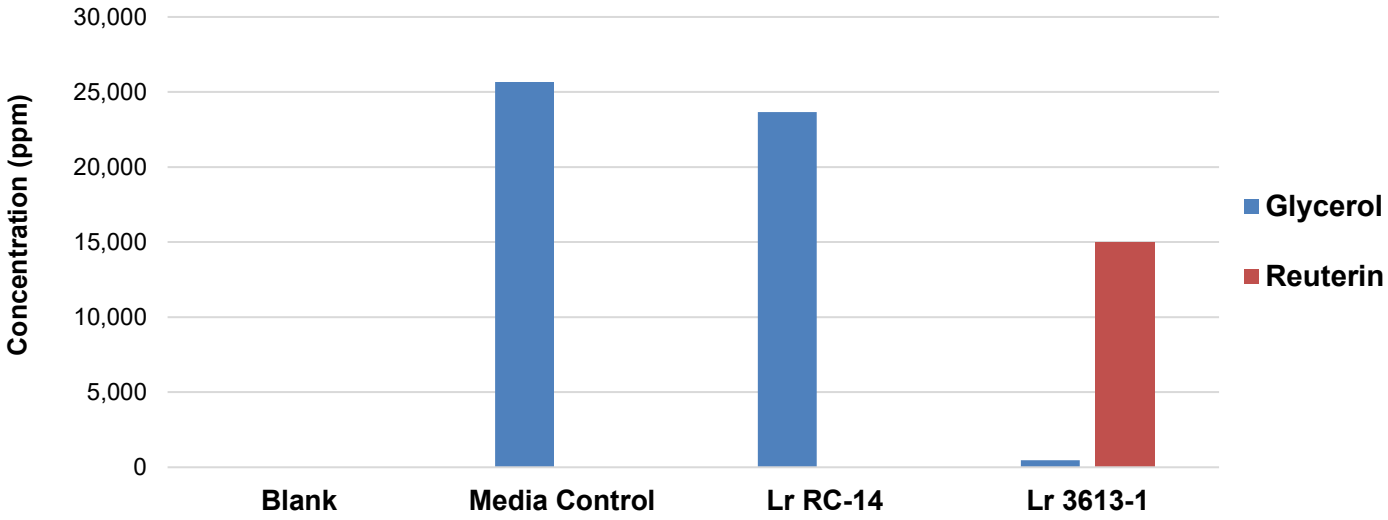


Figure 1. Averages of HPLC Results from UWM**.**

- No glycerol or reuterin was detected in the blank sample run on the HPLC, indicating that there was no background signal that would interfere with the detection of glycerol and reuterin confirming that the analytical method did not generate false positive signals.
- All standards containing known concentrations of glycerol and reuterin were detected and quantified, demonstrating that the HPLC method developed by UWM was successful at detecting and quantifying these compounds using the HPLC.
- Glycerol was detected in all media controls and bacterial culture samples, confirming that glycerol was present in the growth media for the bacteria to metabolize into reuterin.
- Reuterin was not detected in the media control samples, confirming that any reuterin measured in the bacterial supernatant was produced by bacteria and not from the media.
- Reuterin was only detected in the *Lr* 3613-1 supernatant samples, confirming that *Lr* 3613-1 produces reuterin under the conditions evaluated in this study.
- Glycerol concentrations were lower in the *Lr* 3613-1 samples compared to both the media controls and *Lr* RC-14 samples, indicating that *Lr* 3613-1 metabolized a majority of the available glycerol.

Summary

Reuterin was detected in only the *Lr* 3613-1 samples. Glycerol, the precursor for reuterin, was detected in all samples, and was lower in the *Lr* 3613-1 samples. These results suggest that *Lr* 3613-1 converted glycerol into reuterin. The HPLC analysis performed by UWM validated the ability of *Lr* 3613-1 to produce reuterin in the presence of glycerol.

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