

The influence of glycerol upon *L. reuteri* activity against enteropathogens

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BACKGROUND: Probiotics containing *L. reuteri* are popular for treating and preventing bacterial gastrointestinal infections. *L. reuteri*, produces reuterin, an antibiotic that inhibits gram-negative bacteria. Reuterin production is the result of glycerol fermentation by *L. reuteri*. Although *L. reuteri* is normally present in the gastrointestinal system, only small amounts of glycerol are usually available; therefore, the production of reuterin may not occur and this could reduce the effectiveness of the probiotic supplement. Our objective is to identify the minimum concentrations of glycerol required for *L. reuteri* to exert an inhibitory effect on enteropathogenic enterobacteriaceae.

METHOD: Samples containing 10⁸ colony forming units (CFU) of *L. reuteri* DSM17938 (Colikids®, Ache, Brazil/BioGaia, Sweden) were grown with varying concentrations of glycerol (0.05-5%). 10⁶ CFU of *E. coli* (CDC0126/INCQS/FIOCRUZ), *Shigella flexneri* (ATCC/120022), *S. enterica* (ATCC6539) and *Y. enterocolitica* (ATCC9610) were inoculated with *L. reuteri* in the different glycerol concentrations. Each enterobacteria and glycerol 5% without *L. reuteri* cultures were used as positive control groups.

RESULTS: All bacteria were completely inhibited at higher ranges of glycerol concentrations (0.2-5%) and grew at lower concentrations (0.05-0.1%).

CONCLUSION: *L. reuteri* requires at least 0.2% of glycerol to completely inhibit enterobacterial growth. These preliminary findings may influence the current method of use of probiotic supplements. The antibiotic activity of *L. reuteri* may have potential clinical use against important enteropathogens.

KEYWORDS: *L. reuteri*; reuterin; enteropathogenic enterobacteriaceae; glycerol.

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INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC), alongside *Salmonella enterica*, *Shigella flexneri* and *Yersinia enterocolitica*, can be dangerous, especially to communities with low access to proper hygiene and medical care, as they are associated with consumption of food that has been in contact with feces.¹ Enteropathogenic *Escherichia coli* (EPEC) may affect bottle-fed infants because of contaminated water used for rehydration,² and in India, EPEC is responsible for approximately 3% of the diarrheas in children.³ Unfortunately, the overuse of antibiotics has made several types of *E. coli* resistant,

and among 162 diarrheagenic *E. coli* cultures, nearly 86% have been shown to be resistant to Ampicillin, 77% to Chloramphenicol, 30% to Cefuroxime, 24% to Cefotaxime, and a striking 88% to Trimethoprim-Sulfamethoxazole.⁴ Therefore, since common antibiotics are inefficient at inhibiting EPEC, alternative methods have been proposed. For instance, *Lactobacillus reuteri* (*L. reuteri*) can inhibit gram-positive and gram-negative bacteria by producing reuterin, an antibiotic substance created from the organism's fermentation of glycerol. Treatment with *L. reuteri* as a probiotic supplement has been shown to decrease diarrhea in children with mild gastroenteritis.⁵

However, *L. reuteri* is highly fastidious, and must have glycerol available for fermenting, in order to properly grow and produce reuterin. The human gastrointestinal

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tract, however, has low availability of glycerol, since most is enveloped in micelles.⁶ Although several studies show that *L. reuteri* can inhibit the growth of EPEC, what is unknown is whether growing EPEC with larger amounts of glycerol, the inhibitory power of *L. reuteri* will be improved.

The objective of this study is to identify the minimum concentrations of glycerol required for *L. reuteri* to exert an inhibitory effect on enteropathogenic entero-bacteriaceae.

MATERIALS AND METHODS

Prior to initiating studies with *Shigella flexneri* ATCC 120022, *Salmonella enterica* ATCC 6539 and *Yersinia enterocolitica* ATCC 9610, only *L. reuteri* DSM 17938 (equivalent to one drop of COLIKids®, Ache, Guarulhos, Brazil & BioGaia, Stockholm, Sweden) was grown in 3mL of Brain Heart Infusion (BHI) broth with and without glycerol at a concentration of 15% to determine the influence of glycerol on its growth pattern in triplicate. The same was done for enteropathogenic *Escherichia coli* (EPEC) CDC0126 INCQS FIOCRUZ, which was grown with and without glycerol (15%). *L. reuteri* and EPEC were grown together with and without glycerol (15%) (Table 1).

After the preliminary experiment, the second step was to incubate 10^8 CFU of *L. reuteri* DSM 17938 with varying concentrations of glycerol (5%, 2%, 1%, 0.5%, 0.2%, 0.1%, 0.05%) for 24 hours at 35°C.

Then, 10^6 CFU of each of *Escherichia coli* (EPEC) CDC0126 INCQS FIOCRUZ, *Shigella flexneri* ATCC 120022, *Salmonella enterica* ATCC 6539 and *Yersinia enterocolitica*

ATCC 9610 were inoculated individually in the tubes containing *L. reuteri* in different glycerol concentrations, and incubated for 24h at 35°C.

Finally, 10µL of the samples were sown on MacConkey Agar plates and grown for another 24h at 35°C, and colonies were counted (measured in Colony Forming Units - CFU per milliliter). In tubes with BHI broth, each one of the enterobacteria and glycerol at 5% without *L. reuteri* were used as positive control groups.

Temperature of all incubators was set at 35°C. This study was conducted in a Biosafety Level 2 Cabinet.

The T-test was applied comparing the results in Table 1 for *L. reuteri* + EPEC with glycerol (15%) and *L. reuteri* + EPEC without glycerol. The T-test was not applied for any of the results in Table 2, since there was only one replicate of each trial.

RESULTS

As shown in Table 1, when *L. reuteri* is grown with glycerol, EPEC is inhibited with only 9.3 CFU remaining. When it is not grown with glycerol, EPEC grows normally and 10^5 CFU can be noted. There was a significant ($p < 0.001$) difference in EPEC counts after glycerol (15%) was included with *L. reuteri* (Table 1). The inclusion of glycerol makes a significant difference in the inhibition of EPEC.

As shown in Table 2, when all gram-negative bacteria were studied under these conditions and increasing the content of glycerol, they showed the same pattern of inhibition, being completely inhibited at higher ranges of glycerol concentrations (0.2 to 5%) and growing at lower

Table 1 - Initial results evaluating the influence of glycerol on the growth patterns of *L. reuteri* alone, EPEC alone and the combination of both *L. reuteri* and EPEC.

N	With 15% glycerol (in CFU)			Without 15% glycerol (in CFU)		
	EPEC	<i>L. reuteri</i>	<i>L. reuteri</i> + EPEC.	EPEC	<i>L. reuteri</i>	<i>L. reuteri</i> + EPEC.
1	10^6	10^5	$2.8 \times 10^{1*}$	10^6	10^6	10^5 *
2	10^6	10^6	0*	10^6	10^5	10^5 *
3	10^5	10^5	0*	10^5	10^5	10^5 *
Avg.	7.0×10^5	4.0×10^5	9.33*	7.0×10^5	3.67×10^5	10^5 *
p-value						<0.001

N = triplicate number; CFU = colony forming units; Avg. = average; *Count of EPEC CFU; p-value = *L. reuteri* + EPEC with glycerol versus *L. reuteri* + EPEC without glycerol

Table 2 - Colony Forming units/mL of Gram negative bacteria according to glycerol concentrations.

glycerol concentration (%)	CFU/mL of Gram Negative Bacteria						
	5%	2%	1%	0.5%	0.2%	0.1%	0.05%
EPEC	0	0	0	0	0	5.3×10^2	5.7×10^2
<i>S. flexneri</i>	0	0	0	0	0	10^5	$>10^5$
<i>S. enterica</i>	0	0	0	0	0	10^5	$>10^5$
<i>Y. enterocolitica</i>	0	0	0	0	0	0	$>10^5$

*EPEC: Enteropathogenic *Escherichia coli*

concentrations (0.1 to 0.05%) with the exception of *Y. enterocolitica* that only showed growth in glycerol 0.05%.

In all the control tubes, there were 10^6 CFU of each enterobacteria.

■ DISCUSSION

L. reuteri requires at least 0.2% of glycerol to completely inhibit some enteropathogenic enterobacteria growths. Although studies show that reuterin produced by *L. reuteri* exerts an inhibition of bacterial activity,^{7,8} in our study all enteropathogenic bacteria evaluated (EPEC, *Shigella flexneri*, *Salmonella enterica* and *Yersinia enterocolitica*) grew normally in the presence of *L. reuteri*. It was only when glycerol was added to this combination that *L. reuteri* completely inhibited these enteropathogens.

In the human body, concentration of glycerol is quite small. It would be essential to analyze how the manipulated amount of glycerol in our experiment contributed to the levels of inhibition, and what could be expected in the human body. Interestingly, glycerol concentrations ranging from 0.1 - 0.05% were not as efficient as amounts ranging from 0.2% and above. This demonstrates how the addition of glycerol to *L. reuteri* probiotic supplements might be necessary to have a more efficient inhibition of EPEC.

One limitation was that it would be more precise to have counted exactly the number of colonies in some samples (specifically ones that showed $>10^5$ CFU). Another part of the experiment that could show abnormalities are the standard deviation values (SD), due to the fact that results were quantified as $10^{6/5}$, and in these numbers, there is a large difference when calculating an average. However, having a result that is in the range of $10^{6/5}$ indicates normal growth for healthy colonies, a point which SD does not account for.

As all data were collected with the same batch despite different times, it could be considered it would eliminate possible factors such as risking to having damaged or slightly different samples.

■ CONCLUSION

The consequences of the glycerol supplementation in bacterial pathogens of GI tract have yet to be fully explored, as the amount of glycerol available for adequate antimicrobial effect has not been studied. We are introducing a concept that requires further exploration as probiotics do indeed maintain health and good supplementation of natural microbiota.

Glycerol is essential for the antimicrobial activity of *L. reuteri* and reuterin production. These preliminary findings may influence the current method of use of probiotic supplements. It might be wise to consider using

an additional dosage of glycerol when taking *L. reuteri* as a probiotic in order to guarantee a better result of probiotic supplements. Possibly if *L. reuteri* is taken as a probiotic with high glycerol concentrations, then enteropathogens could be inhibited. The antibiotic activity of *L. reuteri* may have potential clinical use against important enteropathogens.

Further work is needed concerning the production of reuterin and other metabolites that may alter probiotic effects and consequently the growth of enteropathogenic bacteria.

■ CONFLICT OF INTEREST

Authors declare no conflict of interest concerning this study.

■ AUTHOR CONTRIBUTION:

Conception and design: Marina Camargo Etchebehere; Collection and assembly of data: Marina Camargo Etchebehere, Cristiane Piveta; Provision of study material: Carlos E. Levy; Data analysis and interpretation: Marina Camargo Etchebehere, Carlos E. Levy; Manuscript writing: Marina Camargo Etchebehere; Final approval of manuscript: All authors.

A INFLUÊNCIA DO GLICEROL SOBRE A ATIVIDADE DE *L. REUTERI* CONTRA ENTEROPATÓGENOS

CONTEXTO: Os probióticos que contêm *L. reuteri* são populares para tratar e prevenir infecções bacterianas do trato gastrointestinal. *L. reuteri* produz reuterina, um antibiótico que inibe bactérias gram-negativas. A produção de reuterina depende da presença de quantidades adequadas de glicerol, cuja fermentação resulta na produção do antibiótico. Embora *L. reuteri* esteja normalmente presente no sistema GI, apenas pequenas quantidades de glicerol estão geralmente disponíveis; portanto, a produção de reuterina pode não ocorrer o que poderia reduzir a eficácia do suplemento probiótico. Nosso objetivo é identificar as concentrações mínimas de glicerol necessárias para que *L. reuteri* exerça um efeito inibitório nas enterobacteriaceas enteropatogênicas.

MÉTODO: 10^8 CFU de *L. reuteri* DSM17938 (Colikids®, Ache, Brasil / BioGaia, Suécia) cresceu com concentrações variáveis de glicerol (0,05-5%). Foram inoculadas 10^6 UFC de *E. coli* (CDC0126 / INCQS / FIOCRUZ), *Shigella flexneri* (ATCC / 120022), *S. enterica* (ATCC6539) e *Y. enterocolitica* (ATCC9610) com *L. reuteri* nas diferentes concentrações de glicerol. Cada enterobacteria e glicerol 5% sem culturas de *L. reuteri* foram utilizadas como grupos de controle positivo.

RESULTADOS: Todas as bactérias foram completamente inibidas em maiores concentrações de glicerol (0.2-5%) e cresceram em concentrações mais baixas (0.05-0.1%).

CONCLUSÃO: *L. reuteri* requer pelo menos 0,2% de glicerol para inibir completamente o crescimento de enterobactéria. Essas descobertas preliminares podem influenciar o método atual de uso de suplementos de probióticos. A atividade antibiótica de *L. reuteri* pode ter potencial uso clínico contra importantes enteropatógenos.

PALAVRAS-CHAVE: *L. reuteri*; reuterina; Enterobacteriaceae enteropatógenas; glicerol.

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