Early Colonization of *Lactobacillus reuteri* after Exposure to Probiotics

Çaglar E*/ Topcuoglu N**/ Ozbey H***/ Sandalli N****/ Kulekci G****

*The **aim** of the present in vivo animal study is to investigate the ability of L. reuteri to colonize the oral flora during infancy. **Study design**: Twenty four rats, aged 1 month, which were pre-analyzed for mutans streptococci and L. reuteri absence in their saliva, were randomly divided into 3 groups. The control group was infected with S. mutans ATCC 25175 at the 2nd month, three times a day for 14 days. S. mutans counts were determined with microbiological saliva analyzes obtained by standard methods of oral swabbing at 3rd, 4th and 5th months. The second group, Probiotic I group, was also infected with S. mutans at the 2nd month, and further infected with L. reuteri ATCC 55730 ($1x10^8$),5 drops per day for 25 days, at the 3rd month. S. mutans and L. reuteri counts were determined at the 3rd, 4th and 5th months. Plates were incubated anaerobically at 37ºC for 48 h, after which colonies were confirmed as L. Reuteri. **Results**: Regarding intra-group analysis, S. mutans counts of the Control group increased steadily during the 3rd. and 4th. months, and a statistically significant ($p<0.05$) reduction was registered at the 5. month. S. mutans counts of the Probiotic I group increased steadily during the 3rd. and 4th. months, and again a statistically significant ($p<0.05$) reduction, parallel with the Control group, was registered at the 5th. month. In the Probiotic II group, S. mutans counts started at a higher level than the Control group and there was a statistically significant ($p<0.05$) reduction of S. mutans at the 5th. month. **Conclusion**: It may be concluded that, L. reuteri promised a better colonization as a 'first colonisation strain'.*

Key words: *Lactobacillus reuteri, probiotic bacteria, rat, saliva, streptococcus mutans.*

INTRODUCTION

Probiotics are live microbial food supplements that may benefit the host influencing the balance between the many species of the commensal flora both in the oral cavity and the rest of the digestive system.[1] *Lactobacillus reuteri* is an obligate heterofermentative resident in the gastrointestinal tract in humans[2] and it is reported to produce antimicrobial substances with a broad spectrum activity, i.e., reuterin[3-4] and reutericyclin.[5] To determine the possible impact of *L. reuteri* ATCC 55730 on early oral colonization, a broad search of the PubMed database was undertaken, using 'probiotics', '*lactobacilli reuteri*', 'oral health', 'caries prevention', 'infancy', 'colonization' and 'children' as index terms. The initial search revealed only two abstracts, one related to colonization and the other to child oral health, work previously done by our research group. The child oral health related paper[6] related oral intervention of *L. reuteri* in children with cleft lip/palate who used a novel probiotic drop. The study had a double-blind, randomized crossover design, where the study group consisted of 19 operated cleft lip/palate children aged 4 to 12 years. The other colonization paper[7] focused on whether *L. reuteri ATCC 55730* could be detected in the oral cavity after discontinuation of administration of a product prepared with this bacterium. At this point the potential role that *L. reuteri* ATCC 55730 might have on infant oral health in relation to early colonization has not been validated yet. Therefore the aim of the present study is to investigate the ability of *L. reuteri* to colonise in the oral flora in infancy *in vivo.*

*Esber Çaglar, Department of Paediatric Dentistry, Dental School, Yeditepe University, Istanbul, Turkey
**Nursen Topcuoglu ,Department of Microbiology, Faculty of Dentistry, Istanbul University, Istanbul, Turke.
***Hilal Ozbey, Department of Paediatric Dentistry, Dental School, Yeditepe University, Istanbul, Turkey.
****Nuket Sandalli, Department of Paediatric Dentistry, Dental School, Yeditepe University, Istanbul, Turkey.
****Guven Kulekci, Department of Microbiology, Faculty of Dentistry, Istanbul University, Istanbul, Turkey.

Send all correspondence to:

Esber Caglar
Department of Pediatric Dentistry, School of Dentistry, Yeditepe University
Bagdat cad 238, Goztepe 34728 Istanbul Turkey
Phone : +90 216 3636044/323
Fax : +90 216 3636211
E-mail: caglares@yahoo.com

MATERİALS AND METHOD

Ethical approval was received from Yeditepe University Experimental Animal Ethics Committee. Animal care was in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The unique study protocol is summarized in Figure 1. Twenty four female Spraque Dawley rats, aged 1 month, which were pre-analyzed for *mutans streptococci* and *L. reuteri* absence in their saliva, were randomly divided into 3 groups.

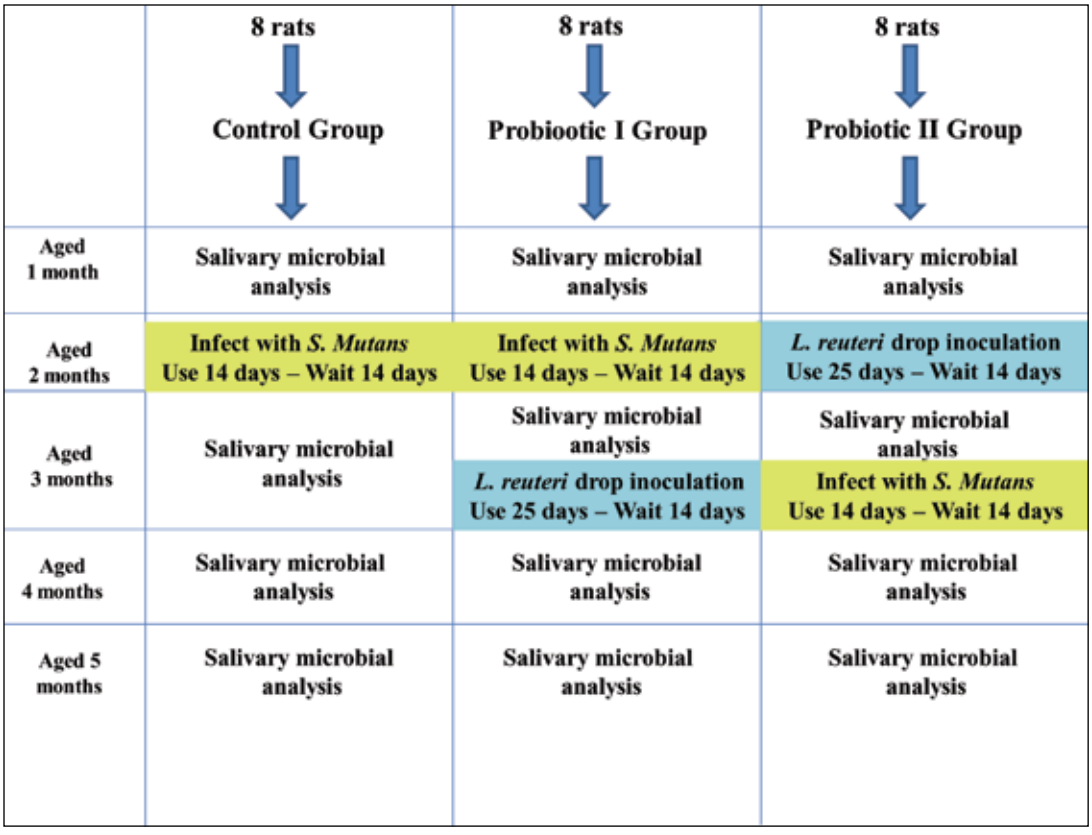
Lactobacillus reuteri ATCC 55730 and *Streptococcus mutans* ATCC 25175 type strains were used throughout the study. Fresh cultures were prepared for each inoculum. Bacteria were cultured overnight at 37°C in the Brain Heart Infusion Broth (BHI, Merck KGaA 64271 Darmstadt, Germany) and used as inoculum. The turbidity of the suspension was adjusted to the McFarland 0.5 turbidity standard.

The control group was infected with *S. mutans* ATCC 25175 at the 2nd month. Approximately 100µl of 10^8 cfu/ml of *S. mutans* (final concentration of about 10^6 cfu/ml) were inoculated using automatic micro-pippettes, three times a day for 14 days. *S. mutans* counts were determined with microbiological saliva analyzes obtained by standard methods of oral swabbing at the 3rd, 4th and 5th months. The second group, Probiotic I group, was also infected with *S. mutans* at the 2nd month, and further infected with *L. reuteri* ATCC 55730 ($1x10^8$) at the 3rd month. The regimen was 5 drops (10µl) of BioGaia Drop® (Eczacıbaşı, Sanico N.V, Belgium) per day for 25 days. *S. mutans* and *L. reuteri* counts were determined at the 3rd, 4th and 5th months. All swab samples were transferred into a vessel with 1 mL of VMG II transport fluid[8], which was tested and found to preserve *L. reuteri* and *S. mutans* counts for 48 h before the study had begun. The samples were transported at room temperature to the laboratory within 24 h. All samples were mixed for 20 seconds for homogenization using a Vortex mixer and were serially diluted with 0.9% saline solution and plated on De Man, Rogosa, Sharpe agar (MRS, Acumedia, Ljusne, Sweden) modified by the addition of 2% sodium acetate and 50 mg/L vancomycin for the cultivation of *L. Reuteri,* and on Mitis Salivarius Bacitracin agar (MSB) (Acumedia Man Inc., Baltimore, Maryland) for the S. mutans counts (detection limits = 10 cfu/ml). Plates were incubated anaerobically (AnaeroGen, Oxoid, Sollentuna, Sweden) at 37ºC for 48 h, after which time colonies were confirmed as *L. reuteri* using a BioGaia AB proprietary method based on reuterin production in the presence of glycerol[7]. The typical colonies were counted and results were expressed as the total number of colony-forming units (cfu/ml).

RESULTS

Regarding intra-group analysis, *S. mutans* counts of the Control group increased steadily at the 3rd. and 4th. months and a statistically significant ($p<0.05$) reduction was registered at the 5th. month. *S. mutans* counts of Probiotic I group increased steadily at the 3rd. and 4th. months and again a statistically significant ($p<0.05$) reduction, parallel with the Control group, was registered at the 5th. month. In Probiotic II group, *S. mutans* counts started at a higher level than the Control group and there was a statistically significant ($p<0.05$) reduction of *S. mutans* at the 5th. month.(Table 1) At this point rats who were inoculated firstly with *L. reuteri* did not show a higher *S. mutans* colonization. *L. reuteri* counts of the Probiotic I

Figure 1. The study protocol



group increased steadily at the 4th. and 5th. months. *L. reuteri* counts of the Probiotic II group increased steadily from the 3rd to the 5th months. *L. reuteri* inoculation and salivary counts are numerated in Table 2 where there are no statistical differences through the 4th and 5th months of Probiotic I group (p=0,889) and the 3rd, 4th and 5th months of Probiotic II group (p=0,206). Regarding inter-group examination there are no statistical differences between 4th and 5th month salivary *L. reuteri* evaluation of Probiotic I and Probiotic II groups (p>0.05). Figure 2 demonstrates *S. mutans* verses *L. reuteri* levels of all groups.

DİSCUSSİON

Oral colonization of probiotic bacteria has been demonstrated in earlier studies[9-16]. In a Finnish study[9] it was reported that although they had withdrawn the use of *L. rhamnosus* (LGG) products, one of their subjects who had received LGG milk at childhood for one year, was found LGG positive in her saliva. The question arose as to whether there was 'permanent colonization of probiotics in childhood?'. In a remarkable study, Taipale *et al* [17] recently stated that 'administration of BB-12 in infancy does not seem to increase or decrease the occurrence of caries by 4 years of age in a low-caries population'.

L reuteri colonization in infancy is clearly unknown. The present animal study projects light onto whether oral colonization of *L. reuteri* in infants takes place at a time when *S. mutans* has not colonized yet. In the present study, groups Probiotic I and II revealed an early increase in *S. mutans* counts and then a total reduction parallel to the control group. It might suggest that *S. mutans* colonization was generally weak due to the dietary regimen (no milk or saccarose feeding was applied regardless of a classic rat dental caries study). Hedberg et al[18] recently stated that *L. reuteri* ATCC PTA 5289 might start a reaction with glucose, lactose and saccarose which would disturb our study protocol. However new studies might take this into account as a weak chain.

Regarding the 1 month after *S. mutans* inoculation comparement of the study groups, there was a statistically difference (p=0,03)

Table 1. *S. mutans* **inoculation and salivary** *S. mutans* **counts**

		3. months cfu/ml	4. months cfu/ml	5. months cfu/ml	Fr	p
Control Group	Mean±SD	155±128,73	177,5±131,99	25±70,71	9,75	* **0,008**
	Median (IQR)	180 (32,5-200)	160 (55-285)	0 (0-0)		
	Geometric Means	94,09	123	0		
Probiotic I Group	Mean±SD	297,5±689,22	427,5±418,56	125±353,55	4,96	* **0,044**
	Median (IQR)	50 (22,5-122,5)	310 (50-750)	0 (0-0)		
	Geometric Means	87,5	365	0		
Probiotic II Group	Mean±SD		390±351,16	25±70,71	Z:-2,53	* **0,011**
	Median (IQR)		300 (200-430)	0 (0-0)		
	Geometric Means		292	0		
P		MW:14,98	KW 2,12	KW 0,01		
		0,001*	0,347	0,994		

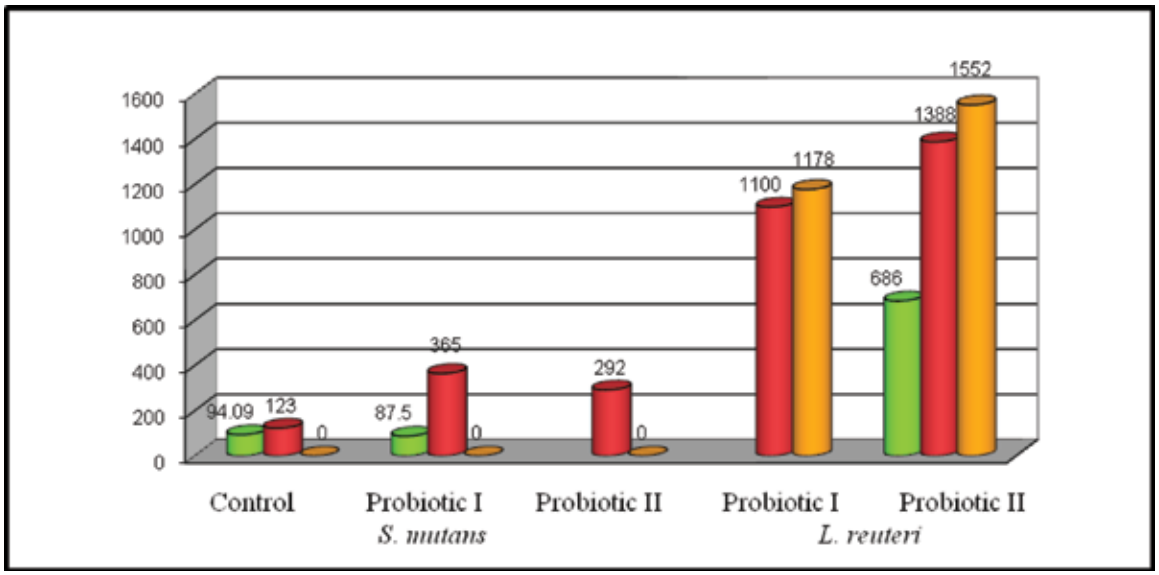
*p<0,05

Table 2. *L.reuteri* **inoculation and salivary** *L.reuteri* **counts**

		3. months cfu/ml	4. months cfu/ml	5. months cfu/ml		p
Probiotic I Group	Mean±SD		1475±1264,63	1750±1581,14	MW:0,14	0,889
	Median (IQR)		1000 (550-2400)	1400 (600-2700)		
	Geometric Means		1100	1178		
Probiotic II Group	Mean±SD	1327,5±1773,59	2500±3312,32	3307,5±3171,46	Fr:3,16	0,206
	Median (IQR)	900 (365-1187,5)	1300 (700-2800)	2600 (500-5600)		
	Geometric Means	686	1388	1552		
MW			0,4	0,71		
P			0,525	0,400		

NS(NOT SIGNIFICANT)

Figure 2. Geometric means of *S. mutans* and *L. reuteri* levels (geometric means)



between the 3rd month *S. mutans* counts of Probiotic I group and the 4th month of Probiotic II and control groups. After 2 months the *S. mutans* inoculation comparement of the study groups revealed statistical differences (p=0,006) between the 4th month *S. mutans* counts of Probiotic I group, the 5th month of Probiotic II and the 4th month of the control groups.

The present study established a framework for *L. reuteri* inoculation after *S. mutans* inoculation (Probiotic I) and *S. mutans* inoculation after *L. reuteri* inoculation (Probiotic II). The target question of what happens to *L. reuteri* colonization in the presence or absence of *S. mutans* is answered by the finding that *L. reuteri* colonization is not affected by the presence of *S. mutans* Our finding is similar to the findings of Comelli *et al* [19] and Tahmourespour *et al* [20].

CONCLUSION

It may be concluded that, *L. reuteri* promises a better colonization as a 'first colonisation strain'. Further studies to ascertain whether *L. reuteri* is a "good survivor colonizer" should be undertaken.

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