http://dx.doi.org/10.5272/jimab.2015214.895

Journal of IMAB
ISSN: 1312-773X
http://www.journal-imab-bg.org

Journal of IMAB - *Annual Proceeding (Scientific Papers)* 2015, vol. 21, issue 4

USE OF THE PROBIOTIC *Lactobacillus reuteri* DSM 17938 IN THE PREVENTION OF ANTIBIOTIC-ASSOCIATED INFECTIONS IN HOSPITALIZED BULGARIAN CHILDREN: A RANDOMIZED, CONTROLLED TRIAL.

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ABSTRACT

Objective: To evaluate the effectiveness of *Lactobacillus reuteri* DSM 17938 for the prevention of antibiotic-associated diarrhoea and *Clostridium difficile*-related infections in hospitalized children in a Bulgarian hospital.

Study design: Children (n=100, aged 3 to 12 years) admitted to the hospital for acute infections were enrolled in a randomized, double- blind, placebo-controlled trial. They were assigned to receive either a probiotic supplement containing 1 x 10^8 CFU *Lactobacillus reuteri* DSM 17938 in the form of one chewable tablet once per day (n=49) (BioGaia AB, Stockholm, Sweden) or placebo (n=48). The probiotic or placebo was taken 2 hours after lunch each day, during the entire period of antibiotic treatment at the hospital and for additional 7 days.

Results: Data from 97 children were included in the final analysis. The incidence of diarrhoea (defined as at least 3 loose or watery stools per day in a 48-hour period that occurred during or up to 21 days after cessation of antibiotic treatment) was unexpectedly low in both groups - *L. reuteri* (n=1) versus placebo (n=1): 2,04 vs. 2,1 per 100 (p>0,05, risk ratio 1,02, 95% CI 0,7-1,4). *L reuteri* DSM 17938 did not significantly affect the incidence or severity of AAD diarrhoea and *Clostridium difficile* infection. We found unusually high colonisation rate of non-symptomatic *C. difficile* measured by toxin-specific ELISA. There was no difference between the probiotic and placebo groups for any of the other secondary outcomes (i.e., incidence of mild diarrhoea, frequency of stool samples positive for *C. difficile* toxin A and B at the beginning and at the end of study period, frequencies of other gastrointestinal symptoms in the same study period) (p<0,05). No adverse events were reported.

Conclusion: Due to the low incidence of antibiotic-associated diarrhoea in both groups, no conclusion can be made on the efficacy of *L. reuteri* DSM 17938 on AAD in hospitalized Bulgarian children. The probiotic did not affect the non-symptomatic high rate of *C. difficile* colonisation (33.3% in the placebo and 38.8% in the *L. reuteri* group at baseline) in this population. There was also no difference between groups regarding different gastrointestinal side effects.

Keywords: Probiotic, Lactobacillus reuteri, Clostridium difficile, antibiotic associated diarrhoea, childhood

EXIBITION

Introduction/background:

Antibiotic associated diarrhoea (AAD) is a side effect of antibiotic treatment and is due to disruption of the autochthonous intestinal flora. It occurs in up to 25% of all individuals receiving antibiotics [1]. Paediatric AAD is less extensively studied but the recent data is that it varies from 6-11% in outpatient clinics to 23-33% in inpatient clinics [2, 3]. In hospitalized patients, AAD is related to significant deterioration of the quality of life, increases in length of stay, cost of medical care and even mortality [4-6] Therefore, identifying strategies to minimize antibiotic-associated diarrhoea could be of significant medical and economic advantage .

Some of the risk factors for the development of AAD include use of broad-spectrum antibiotics, prolonged hospitalization, gastrointestinal surgery and immunosuppression. Disruption of normal gastrointestinal flora, overgrowth of pathogens, and metabolic imbalances are suggested to contribute to altered gastrointestinal function and AAD. The symptoms of AAD range from mild abdominal discomfort to severe diarrhoea and colitis. Colitis may present with abdominal cramping, fever, leucocytosis and faecal leucocytes, hypoalbuminaemia, and colonic thickening. It may progress to severe disease that needs surgical intervention.

Overgrowth of the toxigenic *Clostridium difficile* has been estimated to be the cause in 15-25% of total cases with AAD [7, 8]. Clostridium difficile associated diarrhoea is due to toxin A and B. Clinical symptoms vary from asymptomatic colonization to pseudomembranous colitis, and even death [1, 9, 10]. Other symptoms than diarrhoea and colitis may also occur during or after treatment with antibiotics, such as bloating, stomach pain and other functional abdominal symptoms. In some patients new functional abdominal symptoms occur during and after a course of antibiotics and may last up to four months [11, 12].

Different antibiotics induce different frequencies of AAD, probably due to two factors; the spectrum of activity

of the antibiotic on the gut microbiota as well as the rate of uptake from the gastrointestinal tract. Narrow spectrum antibiotics have low rates of AAD while broad-spectrum have high rates [1].

METHODS

A randomized, double blind, placebo-controlled study was performed between March 2011 and November 2012. The planned investigation was designed to determine the efficacy of *L. reuteri* in preventing AAD. To detect a significant difference in the incidence of diarrhoea and bloating as main outcome parameters using 90% power, 5% level, requires 75 evaluable patients totally. One hundred (100) hospitalized children and adolescents who were prescribed antibiotic treatment for an acute infection other than enterocolitis were recruited on a rolling enrolment basis. Estimating an attrition rate of approximately 25%, the final sample size was calculated to be at least 76 subjects, or 38 subjects in each arm.

The local Ethic Committee at St Marina University Hospital, Varna, Bulgaria, approved the protocol. A written informed consent was obtained from parents before participation in the study.

The guidelines of the Consolidated Standards of Reporting Trials were followed for this randomized controlled trial (RCT). It was registered at ClinicalTrials.gov (NCT01295918).

Hospitalized patients who were at the age of 3 to 12 years, receiving antibiotics for not more than 48 hours, and without antibiotics treatment up to one month prior to enrolment and free from diarrhoea were eligible for entry in the study. Children with three or more soft and unformed or watery stools per day at admission, children receiving chemotherapy or radiation therapy, those with chronic diseases and severe life threatening conditions were excluded from the study. Storage of the study product in a temperature below 25°C during the hot season of the year was ensured. Children were not allowed to consume probiotic containing products throughout the study period.

Eligible patients were randomized to either the treatment or placebo group using a computer generated randomization list of case numbers. Participants entered consecutively starting with the lowest case number in each stratum. Randomisation and labelling of the test-samples were made by an independent physician.

Each patient was assigned to receive either a probiotic supplement containing 1 x 10^8 CFU *Lactobacillus reuteri* DSM 17938 in the form of one chewable tablet once per day (BioGaia AB, Stockholm, Sweden) or placebo, identical in taste and appearance. The probiotic or placebo was taken 2 hours after lunch each day, during the entire period of antibiotic treatment and for an additional 7 days. The unblinding was done when all data were analysed by an independent statistician.

The primary variable was the incidence of diarrhoea during and up to 21 days post antibiotic treatment. An episode of diarrhoea was defined as three or more (e”3) soft and not formed or watery bowel movements per day for at least 48 hours. The secondary variables were as follows: incidence of mild diarrhoea during and up to 21 days after treatment with antibiotics (defined as any soft and not formed or watery bowel movements not fulfilling the definition of AAD), severity of diarrhoea (measured as the total number of soft and not formed or watery bowel movements during an episode of diarrhoea and the presence of blood and mucus in faeces), frequency of stool samples positive for *C. difficile* toxin A and B at baseline, when diarrhoea appears, and at the follow-up 21 days post-antibiotic treatment, frequencies of other gastrointestinal symptoms during the study period (assessed by the validated Gastrointestinal Symptom Rating Score (GSRS score) [13].

A daily diary was used to record the incidence and severity of diarrhoea and the Bristol Stool Scale chart as a tool to assess the consistency of stools (Lewis et al, 1997). Stool samples obtained on admission, on the last day of study period (21 days post antibiotic treatment) and during an episode of diarrhoea were analyzed for *Clostridium difficile* toxin A and B.

Statistical analysis

To detect a significant difference in both the incidence of diarrhoea and severity as main outcome parameters using 90% power, 5% level, required 75 evaluable patients totally. Thus 76 evaluable patients were to be studied. After taking into account that about 20% of participants may not complete the study as planned, it was found that the group size should be 94 (47 subjects per group).

Data were analyzed using the SPSS v.17.0 for descriptive and frequency results and presented as mean ±SD and percentiles. Significance was defined as p value less than 0,05. Pearson ÷² was used to determine whether *L. reuteri* supplementation resulted in a significantly lower incidence of AAD.

Data was analyzed for descriptive and frequency results and presented as mean± standard deviations and percentiles. To determine whether there were differences between groups at baseline, descriptive variables were analyzed using an independent t-test. To determine if there is a difference in incidence of diarrhoea and length of hospital stay between groups a repeat measures analysis was done. To determine whether there were responders versus non-responders (patients within the treatment group who experienced benefit versus those who did not) were categorized. Specific clinical characteristics were compared between responders and non-responders using an independent t-test. Adverse events were identified and analyzed to determine the relationship to probiotic treatment.

RESULTS

A total of 100 patients were enrolled in the study. Three patients were excluded from the data analysis because of lack of compliance. 48 children were assigned to the placebo group and 49 to the probiotic group.

Demographic characteristics such as mean age, sex distribution, and frequency of primary diagnosis, type and number of antibiotics were not statistically different between groups (Table 1).

Table 1. Demographic and clinical characteristics of patients

	Placebo (n=48)	**L. reuteri (n=49)**	**Total**
Sex	M -29 (60,4%) F- 19 (39,6%)	M -26 (53,1%) F- 23 (46,9%)	M -55 (56,7%) F- 42 (43,3%)
Age (years) – mean	8,87(±4,03)	8,82(±3,98)	8,85 (±3,98)
Duration of antibiotic treatment (days)(mean±sd)			
First antibiotic	6,9 (±2,3)	7,59(±2,9)	p>0,05
Second antibiotic	8,25 (±1,67)	7,78 (±1,2)	p>0,05
Third antibiotic	5	9 (±1,73)	
Diagnosis			
Respiratory infections	18 (37,7%)	24 (49%)	NS
Gastrointestinal liver, pancreas infections	4 (29,2%)	9(18,3%)	
Ear, nose throat infections	9(18,8%)	7(14,2%)	
Urinary tract infections	4(8,4%)	4(8,1%)	
Others	3(6,3%)	5(10,2%)	

The prescribed antibiotics are listed in table 2

Table 2.

Antibiotic	Placebo	L. reuteri	Total
Amikacin	2,1%	,0%	1,0%
Cefazoline	39,6%	38,8%	39,1%
Cefotaxime	,0%	2,0%	1,0%
Ceftriaxon	37,5%	46,9%	42,3%
Cefuroxime	6,3%	2,0%	4,1%
Levofloxacin	2,1%	,0%	1,0%
Metronidazol	4,2%	2,0%	3,1%
Piperacillin	8,3%	6,1%	7,2%

The outcome measures are summarised in table 3. We found no difference in the incidence of diarrhoea (e"3 soft and unformed or watery bowel movements per day for at least 48 hours) during the study period in patients ingesting *L. reuteri* (n=1) versus placebo (n=1): 2,04 vs 2,1 per 100 (p>0,05). The episode of diarrhoea in patient ingesting *L. reuteri* was on 18-21 days of participation (11-14 day after antibiotic use and 4-7 days after *L. reuteri* use), with 12 movements/day and the presence of mucus and blood in faeces. The episode of diarrhoea in the patient-ingesting Placebo was during first two days of treatment, without presence of mucus and blood. These two cases with diarroea had negative results of stool samples for *Cl. Difficile toxin A and B.*

As for the secondary outcome-there was also no difference in the incidence of mild diarrhoea (<3 bowel movements for less than 48h) during and after treatment with antibiotics in patients ingesting *L. reuteri* versus placebo, was 0,06 vs. 0,02 mean number of episodes per patient, respectively (p>0,1).

Table 3. Outcome measures

Outcome	Placebo (n=48)	*L. reuteri* (n=49)	RR (95%CI)
Baseline:			
Clostridium difficile toxin A or B			
Positive	16(33,3%)	19 (38,8%)	p>0,1
Negative		32(66,7%)	30(61,2%)
Primary outcome			
Diarrhoea	1(2,04%)	1 (2,1%)	0,98 (0,93-1,02)
Secondary outcome			
Mild diarrhoea			
Incidence (Number of episodes)	2(4,08%)	3(6,3%)	1,5 (1,1-1,9)
Severity (total number of stools)	8	17	
Cl. difficile toxin A or B	15 (31,3%)	14 (28,6%)	

The frequency of stool samples positive for *C. difficile* toxin A or B at the follow up 21 day post antibiotic treatment in patients ingesting *L. reuteri* was 28,6 % (n=14) vs placebo-31,3 % (n=15).At the 21-st day, the baseline positive samples stayed positive in most of the cases in the Placebo group compared to the *L reuteri* group: 43,8% (n=7) and 31,6% (n=6) p>0,1, respectively. In this way the proportion of the samples negative for *Cl. difficile* toxin A and B at day 21, which were positive at baseline was higher in the group taking L. reuteri (68,4%; %; n=13) compared to the Placebo group (56,3%; n=9) p>0,1 There was no relationship between stool samples positive for *Cl. difficile* toxin A and B at baseline and at the end of study and symptoms for diarrhoea (as defined by the diary). The difference in the change to negative test results for *C. difficile toxine* A or B at 21st day from positive test result at baseline between Placebo (56,3%; n=9) and *L. reuteri* (68,4%; n=13) groups is not statisticaly significant.

As for the GSRS symptoms, there were no differences in the frequency or severity of nausea, vomiting, abdominal pain, bloating, heartburn, acid regurgitation, sucking sensations in the stomach, borborygmus, abdominal distension, eructation, flatus, passage of stools, loose stools, hard stool, urgent need for defecation and feeling of incomplete defecation between groups at any visit.

Both the probiotic and placebo were well tolerated and there were no declared adverse events due to the probiotic.

DISCUSSION

There have been recently a growing number of studies supporting evidence about the positive effect of different probiotics for the prevention and treatment [14-18] of different types of diarrhoea in childhood. The effect depends on the type, dose of probiotics and additional clinical characteristics (such as for example nutritional status).

Since the incidence of antibiotic-associated diarrhoea was unexpectedly low in both groups of this study, it provides no evidence of the efficacy of the probiotic *L. reuteri 17938* at a dose of 10^8 per day in the prevention of antibiotic associated diarrhoea, independent of the definition for severity of diarrhoea used. Neither do we prove that it has effect on minor gastrointestinal complaints like abdominal pain, heartburn, acid regurgitation, sucking sensation in the stomach, nausea and vomiting, borborygmus, abdominal distension, eructation, increased flatus, passage of stools, loose stools, hard stools, urgent need for defecation or feeling of incomplete defecation.

The strength of the study includes a large number of recruited patients, adequate randomization, double-blind design, comparable demographic and clinical characteristics at baseline, precise inclusion and exclusion criteria, strict follow-up, optimal length of study, strict adherence to a rigorous protocol, and the excellent compliance rate (less than 3% dropouts).

Weakness of the study is the lack of analysis of faecal *L reuteri DSM 17938* in order to confirm compliance and survival of the probiotic at administration.

It is surprising, however that the number of patients having diarrhoea is so low and that there is a discrepancy between positive stool samples for *Clostridium difficult* toxin A and B and lack of clinical evidence of gastrointestinal signs and symptoms, including diarrhoea. Possible reason could be the mean test sensitivity of the commercially available enzyme immunoassay (EIA) used in the study, which detects toxins A and/or B and range from 72% to 82%, with mean specificity of 97% to 98%. This may lead to an unacceptably low positive predictive value [9].

It is remarkable too that the colonization rate of *Clostridium difficile* at baseline is high (33.3% and 38.8% in the placebo and probiotic group respectively) which could be due to the low toxigenic potential of the strain or low concentration of the toxin.

There are no recent large-scale studies in Bulgaria about the rate of antibiotic-associated diarrhoea and the incidence of asymptomatic *Clostridium difficile* colonisation infection. There is a marked increase in the incidence of *Clostridium difficile* infection and associated mortality across the United States, Canada, and Europe during the last decade [19-21] probiotics could influence the prevalence in some studies- for example in Sweden, in atopic families the preva-

lence of *Clostridium difficile* is high in both groups-supplemented and not supplemented with *L. reuteri*- 80% and 83% at 1 year of age and 73% and 69% at 2 years of age respectively [22]. As a comparison some 10 years earlier the prevalence of *Clostridium difficile* was of 34% in Swedish and 4% in Estonian randomly selected and not supplemented with a probiotic 1-year-old children [23]. In another study from Kanazawa when analysed according to age group, the carriage rates of *Clostridium difficile* were 100, 75.0, 45.5, 24.0, 38.5 and 23.5% in infants and children 0, 1, 2, 3, 4, and 5 years old, respectively [24]

However in Bulgaria there is a general trend of overprescribing antibiotics. On the other hand there is also widely popular frequent consumption on everyday bases of probiotic and prebiotic containing fermented milk products. It is very popular both among children and adults. It might be possible that this traditional diet diminishes the effect of AAD and interferes with the clinically significant signs of diarrhoea associated with *Clostridium difficile* acquisition.

Another reason for the lack of effect could be the type and dose of the probiotic. In a recent study [25] in adults, there has been proven effect for *L. reuteri* supplementation at a dose of $2x10^8$ for 4 weeks which was effective in the prevention of AAD in hospitalized patients. Apart from this irrespective of the definition used, *L. reuteri* significantly reduced diarrhoea incidence in healthy children with lower nutritional status (below-median height-and-weight-for-age z score) [26]. In our study the dose was 10^8 CFU per day and the children recruited were with good nutritional status. The probiotic was taken during the entire period of antibiotic treatment at the hospital and for additional 7 days.

Patients who were invited to the study were not in contact with other hospitalized patients from the ward as this was one of the motivating interventions for families to be compliant with the study protocol. Thus we could have minimized the chances for nosocomial diarrhoea, which was not an outcome for this study.

In our study, we found low prevalence of the AAD and there was no need for preventive measures. Decisions on using probiotics should be based on surveys of the prevalence to avoid unnecessary measures.

CONCLUSION

Due to the low incidence of antibiotic-associated diarrhoea in both groups, no conclusion can be made on the efficacy of *L. reuteri* DSM 17938 on AAD in hospitalized Bulgarian children. The probiotic did not affect the non-symptomatic high rate of *C. difficile* colonisation *(*33.3% in the placebo and 38.8% in the *L. reuteri* group at baseline) in this population. There was also no difference between groups regarding different gastrointestinal side effects.

Acknowledgements

The clinical trial has been supported by a grant from BioGaya AB, Sweden, which was distributed between authors and the institution where the investigation took place - University Hospital St. Marina, Varna, Bulgaria. The Clinical trial grant was intended to support clinicians in carrying out the clinical trial study.

We should like to thank prof. Andras Arato and prof. Raanan Shamir for their critical review of the manuscript and invaluable comments. In addition, sincere thanks must go to Dr. Milena Sirakova for her suggestions in order to improve the quality of the manuscript.

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Please cite this article as: Georgieva M, Pancheva R, Rasheva N, Usheva N, Ivanova L, Koleva K. Use of the probiotic Lactobacillus reuteri DSM 17938 in the prevention of antibiotic-associated infections in hospitalized Bulgarian children: a randomized, controlled trial. *J of IMAB.* 2015 Oct-Dec;21(4):895-900. DOI: http://dx.doi.org/10.5272/jimab.2015213.879

Received: 22/07/2015; Published online: 07/10/2015

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