A randomised, double-blind, placebo-controlled clinical trial on the tolerance of dietary supplementation with *Lactobacillus reuteri* ATCC PTA 6475 in adults

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Introduction

Lactobacilli are commensal bacteria commonly found in the gut of man, which are traditionally used in food preservation and are considered safe for human consumption. The established safe use of added lactobacilli in a diversity of foods and supplement products worldwide supports this conclusion (Borriello et al., 2003; Reid et al. 2006). *L. reuteri* is one species of *Lactobacillus* that naturally inhabits the gastrointestinal tract of humans and is one of the few autochthonous (indigenous) *Lactobacillus* species in infants as well as adults (Reuter, 2001, Oh et al., 2010).

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). The development of promising bacterial strains as candidate probiotics has been discussed in several fora and the unequivocal identification of the strain, the demonstration of a lack of antibiotic resistance not belonging to the wild-type of the species and the need for an initial evaluation of safety and colonisation by such candidates in humans are common conscensus outcomes (Vankerckhoven et al., 2008).

L. reuteri DSM 17938 (formerly *L. reuteri* ATCC 55730; Rosander et al., 2008) is a strain that has been widely studied in clinical trials and has been shown to have probiotic, health-promoting effects in both adults and children (Connolly 2004; Casas & Dobrogosz, 2000). This strain has been shown in numerous studies to be safe for human consumption and it has been shown to colonise the human gastrointestinal tract (Valeur et al., 2004). After consumption, this strain survives passage through the human gastrointestinal tract and appears as live bacteria in the feaces (e.g. Wolf et al., 1995; Rosander et al., 2008).

Lactobacillus reuteri ATCC PTA 6475 was originally isolated from the breast-milk of a Finnish mother in 1997. The strain, originally isolated by researchers at BioGaia was originally designated as *L. reuteri* "MM4-1A" (Mother's Milk from mother number 4, first sample, clone A). The strain was deposited at American Type Culture Collection on December 21st 2004 and given the designation *Lactobacillus reuteri* ATCC PTA 6475. *L. reuteri* ATCC PTA 6475 has been shown to have 99% similarity with the *L. reuteri* type strain using 16SrRNA analysis (BioGaia, 2003) and 16S rDNA analysis (Egervärn et al., 2007).

Screening (internal data at BioGaia) have shown that *L. reuteri* ATCC PTA 6475 has a typical API sugar fermentation pattern of the *L. reuteri* species, has similar intestinal mucus binding properties to those of *L. reuteri* DSM 17938, has greater acid tolerance in synthetic stomach juice than *L. reuteri* DSM 17938 (which has been shown to colonise the human stomach; Valeur et al., 2004), and is free from plasmids.

The strain was also screened for potential anti-inflammatory activity and found to be one of only very few *L. reuteri* strains that has the ability to reduce the production of the inflammatory cytokine TNF_{alpha} in toxin-stimulated human macrophages. (Lin et al. 2008) and ca modulate NF-κB and MAPK signalling in human myeloid cells (Iyer et al., 2008) The strain forms extensive and robust biofilms in which the bacterium retains its anti-inflammtory activity (Jones & Versalovic, 2009).

This study was preformed to confirm that *L. reuteri* ATCC PTA 6475 is well-tolerated in humans.

Methods

This was a randomised, double-blind, placebo-controlled clinical trial to assess the safety and tolerance of a mixture of *Lactobacillus reuteri* strains in healthy volunteers. The primary objective was to demonstrate that the strain *L. reuteri* ATCC PTA 6475 alone or in combination with *L. reuteri* DSM 17938, is well-tolerated in humans, measured as a lack of difference in blood safety parameters and symptom reporting before and after ingestion compared to placebo.

Twenty subjects, were recruited during the first half of 2008 from the local area around the clinic in Bari, Italy after obtaining their written informed consent. Inclusion criteria were as follows: age 18–65 years, written informed consent, stated availability throughout the entire study period, healthy, i.e. without any major illnesses (allergic symptoms however, both skin and respiratory, were acceptable), and mental ability to understand and willingness to fulfil all the details of the protocol. Exclusion criteria were: significant disease as decided by the Principle Investigator, pregnancy, use of oral antibiotics during 2 weeks prior to ingestion of the study product and participation in other clinical trials.

After inclusion each subject was randomised to one of 3 groups:
- *L. reuteri* Progastria standard dose: *L. reuteri* ATCC PTA 6475 ($7.0x10^8$ CFU/day) + DSM 17938 ($7.7x10^8$ CFU/day; 5 subjects)
- *L. reuteri* high dose: *L. reuteri* ATCC PTA 6475 ($1x10^{11}$ CFU/day; 8 subjects)
- Placebo (7 subjects)

A baseline analysis consisted of sampling of fasting blood for analysis and completion of a questionnaire related to the incidence of gastro-intestinal symptoms was performed.

Subjects were then asked to take 1 sachet of blinded study product each day for 28 days. Sachets were kept refrigerated at all times up to the point of consumption to ensure that there were no losses in bacterial viability during the study. The subjects were instructed in writing to open the sachet immediately before consumption, pour the powder into a glass containing cold water (at least 100ml), stir well for 15 sec and swallow directly.

Throughout the study, the contents of the study products were unknown to both the subjects participating and to the principle investigator. The study code was held by the formulator at BioGaia AB (Lund, Sweden) and was not revealed until the completion of all analyses and compilation of the database at the end of the study.

Study product

Since a new strain was used that had not been incorporated into product matrices at the time of this study, the study product consisted of freeze-dried powder containing the bacteria mixed with a sucrose/maltodextrin carrier (1.5g) packed in a sachet. Placebo study product contained only the carrier and no added bacterial culture although analysis of the placebo sachets showed that they contained a small in production contamination of 774 CFU of detectable *L. reuteri.* This dose was considered too small to have any effect on the placebo test subjects as it is 6-9 log lower levels than in the active products and was thus acceptable.

The study products were kept refrigerated at all times during the study and the participants were informed of this when the study product was given to them. Paralell stability studies by the supplier confirmed no losses of viability during the study period.

Dose rationale
The expected dose for human use in the future is $1x10^8$ CFU/day on the last day of shelf-life (based on experience with *L. reuteri* ATCC 55730/DSM 17938) but since the viability of *L. reuteri* decreases upon storage of a product, the maximal dose after production to which a consumer may be exposed is $1x10^9$ CFU per unit product. This was thus chosen as the target "standard" dose. The standard dose wsa a mixture of the strains to mimic the proposed future product composition.

The higher dose is 100-fold the lower dose in accordance with early safety studies with *L. reuteri* ATCC 55730 (Wolf et al., 1995). Since this dose is given to determine tolerance of *L. reuteri* ATCC PTA 6475, only this strain was added to the study product.

Blood analyses
Fasting venous blood samples were drawn at baseline and after 4 weeks of supplementation. The blood samples were analyzed by standard procedures at Clinical Chemistry Laboratory of Bari University Hospital for the following parameters: Fe, haemoglobin, blood corpuscle volume (MCV), white blood cell count (WBC), cholesterol, triglycerides (TG), albumin, total protein, glucose, calcium, sodium, potassium, total bilirubin, ALAT, ASAT, GT, creatinine, urea, urate and C-reactive protein.

Health questionnaire
After randomisation volunteers were given personal diaries (Table 2) and were asked to fill in the first (baseline) diary before leaving and the second, third, fourth and fifth diaries on Day 7, 14, 21 and 28 respectively, to document their experience during the previous 7 days.

Compliance
Subjects were asked to return the empty sachets from their study product after Day 28. These were used to assess compliance together with a question in the diary (Table 2).

Ethical approval
The study was approved by the Ethical Committee of Bari University Hospital prior to start.

Statistical analysis
Where there were obvious or potential differences between means in blood variables at baseline compared to those on Day 28, significance was analysed using Student´s t-test.

Results
No subjects failed to complete the study and reported compliance from the diaries showed 98%, 96% and 94% in the placebo, standard dose and high dose groups, respectively. There was extensive doumentation of potential adverse events related to the gastro-intestinal tract and general health via the diaries. Other than these reports, there were no other adverse events reported by the participants during the study indicating a good general tolerance of the study products.

Blood analysis
Blood parameters were generally unchanged by supplementation with any of the study products as shown by a comparison of Day 0 values with Day 28 values (Table 1). In the placebo group there was a significant increase of total protein and albumin during the 28 day study whilst in the *L. reuteri* high dose group, significance was reached for higher sodium

levels on Day 28. However, these changes are within normal ranges and they are thus unlikely to represent more than normal variation (Table 1).

Health questionnaire

The questionnaire has been used in earlier saftey and tolerance clinical trials using *L. reuteri* and Table 3 shows the variability in reporting at baseline of the earlier Swedish subjects in reference studies 1 and 2 compared to the Italian population in the present study. Whilst nausea, stomach ache, gases and heartburn were represented in the Swedish population, these were absent at baseline in the present Italian study population. Constipation was however, common in the present study group at baseline (Table 3).

Subjective symptom scoring of health related parameters and general well-being during the study are shown in Tables 4-6. There was essentially no reported incidence of fever, nausea, vomiting stomach ache and heartburn in the study subjects of any group. The number of reported sick days was highest in the high dose group but similar to that reported in the placebo group, whilst the standard dose group did not report any sick days. Diarrhoea incidence was highest in the placebo group but not reported at all in the high dose group. Constipation was common in all groups from baseline, before supplementation and throughout the trial. The incidence of reported abdominal gas was similar between the placebo and high dose groups, but lower in the standard dose group.

In summary, these reported gastro-intestinal and health symptoms indicated that the administration of *L. reuteri* ATCC PTA 6475 at doses up to $1x10^{11}$ CFU/day during a 28 day period was well tolerated and did not induce any untoward or unexpected adverse events in the study subjects.

Discussion

Safety evaluation of a new bacterial strain in humans is the result of experience in a wider use in the human population. However, initial tolerance studies such as that presented here are important even though they are performed in a very limited study group. Human *L. reuteri* DSM 17938 has been shown by extensive use in humans to be safe and well-tolerated and has been GRAS approved (see *www.accessdata.fda.gov/scripts/fcn/gras_notices/808166A.PDF*). The main objective of this trial was to gain experience with a further *L. reuteri* strain ATCC PTA 6475 which has not been studied in humans earlier. The results indicate that the use of *L. reuteri* ATCC PTA 6475 in combination with *L. reuteri* DSM 17938 at the dose predicted to have clinical efficacy, was well-tolerated. Further, 100-fold higher doses of the *L. reuteri* ATCC PTA 6475 were also well-tolerated in humans.

Faecal sampling for the presence of the previously undocumented strain *L. reuteri* ATCC PTA 6475 was not performed in this study. *L. reuteri* ATCC PTA 6475 is planned for use in a combination product with *L. reuteri* DSM 17938 where the main target is the control of *H. pylori* infection in humans. Since *H. pylori* infects the human gastric and upper duodenal mucosa, there is no reason to demand full gastro-intestinal passage of the strain to predict efficacy. Later studies will examine the more relevant colonisation of the human gastric mucosa using biopsy material from this site.

Conclusions

L. reuteri ATCC PTA 6475 in combination with *L. reuteri* DSM 17938 at the dose predicted to have clinical efficacy, was well-tolerated in humans given supplementation daily for 28 days. Further, 100-fold higher doses of the *L. reuteri* ATCC PTA 6475 were also well-

tolerated in humans under 28 day use. Based on these data and experience from clinical studies of *L. reuteri* DSM 17938, this new *L. reuteri* strain is safe to use for further study in humans up to doses of $1x10^{11}$ CFU/day.

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Table 1. Blood parameters

	Placebo		Progastria low dose		Progastria high dose		Normal range
	Day 0	***Day 28***	***Day 0***	***Day 28***	***Day 0***	***Day 28***	
Parameter							
GT (U/L)	35±23	31±15	35±13	27±13	37±13	40±14	8-29
ASAT (U/L)	32±11	30±12	27±7	37±9	43±10	44±8	20-50
ALAT (U/L)	18±6	21±8	20±10	20±8	24±4	24±7	20-39
Creatinine (mg/dl)	0.68±0.23	0.59±0.25	0.74±0.27	0.62±0.26	0.75±0.26	0.74±0.25	0.2-1.0
Urea (mg/dl)	32±5	30±10	18±10	18±6	22±11	23±11	25-40
Bilirubin (mg/dl)	0.57±0.31	0.52±0.25	0.36±0.30	0.30±0.18	0.49±0.21	0.40±0.15	0.1-1.0
Haemoglobin (g/dl)	13.7±1.7	13.7±1.5	14.7±1.6	14.6±1.5	14.9±1.1	15.0±0.8	11-14
White blood cells (10^9/L)	6.35±1.22	5.71±1.08	6.95±1.62	7.15±0.96	6.00±1.16	6.39±0.81	5.24-9.79
CRP (md/dl)	3.4±1.3	3.2±2.0	3.1±2.1	9.6±17.0[a]	2.6±2.1	3.2±1.5	<7
MCV (fL)	78.0±8.3	78.7±8.0	82.0±6.7	81.4±8.6	83.1±6.8	83.5±5.3	80-94
Sodium (mmol/L)	140±5	138±5	144±3	139±7	135±2	143±6**	136-146
Potassium (mmol/L)	3.8±0.4	4.0±0.5	4.3±0.6	4.3±0.5	4.0±0.4	3.9±0.4	3.0-4.5
Urate (mg/dl)	4.3±1.4	4.4±1.0	5.4±1.7	6.0±1.0	4.2±1.4	4.3±0.9	2.2-6.6
Calcium (mg/dl)	9.1±0.6	8.7± 0.6	9.2±0.6	9.1± 0.8	8.8±0.6	8.9± 2.0	8.8-10.8
Iron (µg/dl)	89±30	92±24	75±40	84±31	91±32	89±29	53-119
Glucose (mg/dl)	83±11	86±10	83±11	86±10	84±7	89±9	74-106
TG (mg/dl)	118±27	121±30	118±26	117±45	119±37	112±30	32-158
Cholesterol (mg/dl)	165±22	161±19	157±16	157±21	165±19	167±21	M: 114-198 F: 125-212
Total protein (g/dl)	6.2±0.4	7.0±0.6*	7.1±0.5	6.5±0.6	6.4±0.6	6.2±0.8	7.0-8.2
Albumin (g/dl)	3.4±0.3	3.9±0.3*	3.9±0.2	3.6±0.4	3.6±0.3	3.4±0.5	3.0-5.5

Means ± SD from 7, 5 and 8 subjects in the placebo, low dose and high dose groups, respectively. [a]One person had a outlying value of 40.
T-test (2-sided): Day 0 versus Day 28; *P<0.05, **P<0.01.

Table 2. Diary questionnaire

The study subjects were asked to fill in this diary at baseline, before the start of study product, and again after 1, 2, 3 and 4 weeks of supplementation. *1-7 indicates choice of 1, 2, 3, 4, 5, 6 or 7 could be made.

1. How has your general health been in the last week?	*Normal*	*Poor*
2. Have you had fever?	*Yes*	*No*
If yes, how many days?	*1-7**	
3. How many days have you been sick/absent from work/studies in the past week?		
Number of days?	*0-7*	
4. Have you had diarrhoea (> 3 bowel movements/day)	*Yes*	*No*
If yes, how many days?	*1-7*	
5. Have you had any days without bowel movement?	*Yes*	*No*
If yes, how many days?	*1-7*	
6. Have you felt nausea?	*Yes*	*No*
If yes, how many days?	*1-7*	
7. Have you vomited in the last week?		
If yes, how many days?	*1-7*	
8. Have you had stomach ache?	*Yes*	*No*
If yes, how many days?	*1-7*	
9. Have you had unusually high amounts of flatulence?	*Yes*	*No*
If yes, how many days?	*1-7*	
9. Have you had heartburn ?	*Yes*	*No*
If yes, how many days?	*1-7*	
10. Have you taken the study product every day the last 7 days?	*Yes*	*No*
If no, how many times did you miss taking it?		*1-7*

Table 3. Baseline reporting of health questionnaire in whole study group (n=20).

Question (see Table 2)	Present study	Reference study 1*	Reference study 2**
Number of study subjects	20	16	16
1. Number of subjects reporting "poor" health	0	0	0
2. Number of subjects reporting days with fever	0	0	1
Average duration of fever (days)	-	-	1
3. Number of subjects reporting sick days	1	0	0
Average duration of sick leave (days)	1	-	-
4. Number of subjects reporting diarrhoea	1	1	0
Average duration of diarrhoea (days)	1	2	-
5. Number of subjects reporting constipation	7	3	1
Average duration of constipation (days)	1.7	2	1
6. Number of subjects reporting nausea	0	2	0
Average occurrence of nausea (days)	-	2.3	-
7. Number of subjects reporting vomiting	0	0	0
Average occurrence of vomiting (days)	-	-	-
8. Number of subjects reporting stomach ache	0	4	2
Average occurrence of stomach ache (days)	-	2	1.5
9. Number of subjects reporting abnormal gas	0	3	4
Average occurrence of abnormal gas (days)	-	2.6	2
10. Number of subjects reporting heartburn	0	2	1
Average occurrence of heartburn (days)	-	4.5	4

The answers indicate the occurrence of symptoms during the previous 7 days. The average number is based on the reporting of only the subjects with symptoms.

* BioGaia Internal Report EC033 R&D, 2006, based on a study of 16 Swedish subjects

* BioGaia Internal Report EC040 R&D, 2008, based on a study of 16 Swedish subjects

Table 4. Health questionnaire outcome – Placebo group (n=7).

Question (see Table 2)	Base	D1-7	D8-14	D15-21	D22-28	Incidence
1. Number of subjects reporting "poor" health	0	0	0	0	0	0
2. Number of subjects reporting days with fever	0 (1)	0 (1)	0	1	0	0.14
Average duration of fever (days)	-	-	-	1	-	
3. Number of subjects reporting sick days	0	1 (2)	3	0	1(3)	0.71
Average duration of sick leave (days)	-	2	1	-	2	
4. Number of subjects reporting diarrhoea	0	0 (1)	2	0	2	0.57
Average duration of diarrhoea (days)	-	-	1	-	1	
5. Number of subjects reporting constipation	2	2	0 (2)	0	1	0.42
Average duration of constipation (days)	1.5	1	1	-	1	
6. Number of subjects reporting nausea	0	0	0	0	0	0
Average occurrence of nausea (days)	-	-	-	-	-	
7. Number of subjects reporting vomiting	0	0	0	0	0	0
Average occurrence of vomiting (days)	-	-	-	-	-	
8. Number of subjects reporting stomach ache	0	0	0	0	0	0
Average occurrence of stomach ache (days)	-	-	-	-	-	
9. Number of subjects reporting abnormal gas	0	2 (1)	2	1	0 (1)	0.71
Average occurrence of abnormal gas (days)	-	2	2	1	-	
10. Number of subjects reporting heartburn	0	0	0	0	0	0
Average occurrence of heartburn (days)	-	-	-	-	-	
11. Compliance (% sachets taken)	-	100	98	100	94	

Subjects were asked to assess the previous 7 days in their responses. The averages coupled to each question represent the mean of the responses of only those reporting the symptom.
Numbers in parentheses indicate the number of persons with missing information.
Incidence = number of events/person Day 1-28.

Table 5. Health questionnaire – *L. reuteri* Progastria Standard dose group (n=5).

Question (see Table 2)	Base	D1-7	D8-14	D15-21	D22-28	Incidence
1. Number of subjects reporting "poor" health	0	0	0	0	0	0
2. Number of subjects reporting days with fever	0	0	1	0	0	0.2
Average duration of fever (days)	-	-	1	-	-	
3. Number of subjects reporting sick days	1	0(1)	0	0	0	0
Average duration of sick leave (days)	1	-	-	-	-	
4. Number of subjects reporting diarrhoea	0	1	0 (1)	0	0	0.2
Average duration of diarrhoea (days)	-	1	-	-	-	
5. Number of subjects reporting constipation	3	3	2	1	1 (1)	1.4
Average duration of constipation (days)	1.7	1.7	1	2	3	
6. Number of subjects reporting nausea	0	0	0	0	0	0
Average occurrence of nausea (days)	-	-	-	-	-	
7. Number of subjects reporting vomiting	0	0	0	0	0	0
Average occurrence of vomiting (days)	-	-	-	-	-	
8. Number of subjects reporting stomach ache	0	0	0	0	0	0
Average occurrence of stomach ache (days)	-	-	-	-	-	
9. Number of subjects reporting abnormal gas	0	0	1	0 (1)	0	0.2
Average occurrence of abnormal gas (days)	-	-	1	-	-	
10. Number of subjects reporting heartburn	0	0	0	0	0	0
Average occurrence of heartburn (days)	-	-	-	-	-	
11. Compliance (% sachets taken)	-	100	94	91	100	

Subjects were asked to assess the previous 7 days in their responses. The averages coupled to each question represent the mean of the responses of only those reporting the symptom. Numbers in parentheses indicate the number of persons with missing information. Incidence = number of events/person Day 1-28.

Table 6. Health questionnaire – *L. reuteri* ATCC PTA 6475 high dose group (n=8)

Question (see Table 2)	Base	D1-7	D8-14	D15-21	D22-28	Incidence
1. Number of subjects reporting "poor" health	0	0	0	0	0	0
2. Number of subjects reporting days with fever	0	0	0	0	1 (1)	0.13
Average duration of fever (days)	-	-	-	-	1	
3. Number of subjects reporting sick days	0	2	2	3	2	1.13
Average duration of sick leave (days)	-	1.5	1.5	1.3	1	
4. Number of subjects reporting diarrhoea	1	0	0	0	0 (2)	0
Average duration of diarrhoea (days)	1	-	-	-	-	
5. Number of subjects reporting constipation	2	2	2	3	1 (3)	1.00
Average duration of constipation (days)	2	2.5	1	1.3	1	
6. Number of subjects reporting nausea	0	0	0	0	0	0
Average occurrence of nausea (days)	-	-	-	-	-	
7. Number of subjects reporting vomiting	0	0	0	0	0	0
Average occurrence of vomiting (days)	-	-	-	-	-	
8. Number of subjects reporting stomach ache	0	0	0	0	0	0
Average occurrence of stomach ache (days)	-	-	-	-	-	
9. Number of subjects reporting abnormal gas	0	2	1	1 (2)	2 (3)	0.75
Average occurrence of abnormal gas (days)	-	1.5	2	2	1	
10. Number of subjects reporting heartburn	0	0	0	0	0	0
Average occurrence of heartburn (days)	-	-	-	-	-	
11. Compliance (% sachets taken)	-	95	93	89	98	

Subjects were asked to assess the previous 7 days in their responses. The averages coupled to each question represent the mean of the responses of only those reporting the symptom. Numbers in parentheses indicate the number of persons with missing information. Incidence = number of events/person Day 1-28.