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PEDIOCOCCUS PENTOSACEUS CECT 8330 AND BIFIDOBACTERIUM LONGUM CECT 7894 SHOW A TREND TOWARDS LOWERING INFANTILE EXCESSIVE CRYING SYNDROME IN A PILOT CLINICAL TRIAL

JONATHAN SANTAS[1], MARI CARMEN FUENTES[1], RAMON TORMO[2], RAFAEL GUAYTA-ESCOLIES[3], ELISABET LÁZARO[1] AND JORDI CUÑÉ*[1]

[1]AB-Biotics, S.A., Edifici Eureka, Campus UAB, 08193, Bellaterra, Barcelona, Spain.
[2]Gastroenterology Pediatric Service, Quirón Hospital, Barcelona, Spain.
[3]Pharmaceutical Association of Catalunya, Barcelona, Spain.

ABSTRACT

The aim of this study was to evaluate the *in vitro* probiotic properties of *Pediococcus pentosaceus* CECT 8330 and *Bifidobacterium longum* CECT 7894 and their suitability as candidates for treating infantile excessive crying syndrome. Results reveal that *P. pentosaceus* is able to induce IL-10 production, and the combination with *B. longum* CECT 7894 shows a broad spectrum inhibitory activity against pathogens. *P. pentosaceus* CECT 8330 and *B. longum* CECT 7894 were combined in a single formula which was confirmed to be well tolerated in a pilot, randomized, double-blind clinical trial in infants with excessive crying syndrome. Moreover, a trend towards a greater reduction in daily crying time was observed in the probiotic group compared to placebo (81.0 ± 11.2 vs 54.1 ± 8.6 reduction in minutes per day, respectively; P=0.083). Given the small sample size, these analyses should be repeated in a larger study.

KEYWORDS: ***Pediococcus pentosaceus*, *Bifidobacterium longum*, *Lactobacillus reuteri*, infant colic, excessive crying.**

*** Corres ponding author**

JORDI CUÑÉ
AB-Biotics, S.A., Edifici Eureka, Campus UAB, 08193, Bellaterra, Barcelona, Spain

INTRODUCTION

Excessive crying syndrome, most commonly referred to as infant colic, is one of the most common causes of visits to paediatricians during the first 3-4 months of an infants' life[1]. This condition can have serious consequences for family quality of life and infants whose crying persists beyond 3 months of age may have a higher risk of adverse outcomes in later years[2,3].Despite the potential negative effects of infant colic on infant and parental health, no consensus has been reached on its exact definition, nor on its treatment[4]. Colic is variously referred to as unexplained and inconsolable crying, which causes distress to parents. One of the most used definitions is based on a time rule (modified Wessel's criteria[5]): more than 3 hours of crying per day for 3 or more days for at least 1 week, although in daily clinical practice a significant portion of parents will be unable to permit this threshold of crying time. Recent data supports the concept that aberrant gut microbiota can be one of the main causative factors of infant colic, which can influence gut intestinal inflammatory status, gas production, and motor function[6]. For instance, bacteria such as *Clostridium difficile*[7] and gram-negative gas-producing species related to *Escherichia* and *Klebsiella*[6] have been positively associated with infant colic. This hypothesis has generated substantial research interest in the role of probiotics as a promising management option for modulating gut microbiota, conferring health benefits[8] and thus, improving crying outcomes. Some clinical trials have examined the beneficial effects of probiotics for treating infant colic, and although some of them have reported that probiotic administration can improve symptoms of infant colic[9,10], others have failed to demonstrate a positive effect[11]. This heterogeneity could be explained, at least in part, by the insufficient spectrum of efficacy of current probiotic formulas and by use of open-label design in several studies [9,12,13]. Moreover, evidence suggests that current probiotics may decrease crying time in exclusively breastfed infants with colic, but do not support the same effect on formula-fed infants[14], who are known to develop a different microbiota compared to their breastfed counterparts[15]. Therefore, further studies are necessary to design probiotic products specifically aimed at treating infant colic. The main objective of this study was to perform an *in vitro* screening for selecting LAB with probiotic properties of interest for treating infant colic, and to evaluate its tolerability and efficacy in a pilot clinical trial. These preliminary data permitted estimating an appropriate sample size for future studies.

MATERIALS AND METHODS

(i) In vitro probiotic properties

Pediococcus pentosaceus CECT 8330 and *Bifidobacterium longum* CECT 7894 strains were selected among those resistant to gastrointestinal conditions from a previous screening of LAB isolated from fresh stools of healthy children[16]. *Lactobacillus reuteri* DSM 17938 from BioGaia® (Sweden), usually recommended as a probiotic treatment for infant colic[17], was used as a control for comparative purposes. *In vitro* adhesion assay to mucus and intestinal epithelial cells was performed as described by Collado et al[18]. The ability of bacterial strains to induce IL-10 production was assayed by using human monocytic THP-1 cell line differentiated into macrophages. The capacity to inhibit the growth of intestinal pathogens was studied against *Escherichia coli* ATCC 10538, *Enterobacter aerogenes* ATCC 13048, *Klebsiella oxytoca* KT 801, *Bacteroides vulgatus* ATCC 8482, *Enterococcus faecalis* ATCC 29212, and *Clostridium difficile* ATCC 9689. Gas produced from fermentation was qualitatively assayed for *P. pentosaceus*

CECT 8330, *B. longum* CECT 7894, and *L. reuteri* DSM 17938, as described by Pilone et al.[19] Amine production of *P. pentosaceus* CECT 8330 and *B. longum* CECT 7894 was determined as described by Bover-Cid et al.[20], and the production of D-/L-lactic acid by using D-/L-Lactic Acid Enzymatic test from Boehringer Mannheim/R-Biopharm. The potential toxicity of *P. pentosaceus* CECT 8330 was assayed in neonatal rats receiving 5 ml/kg of daily prepared *P. pentosaceus* CECT 8330 ($2.5x10^{10}$ CFU/kg) for 5 days or vehicle (water). After the last day of administration, 1 treatment group and 1 control group were euthanized. Necropsy was conducted and liver collected for bacterial translocation analysis. Other observations included: morbidity/mortality, body weight and clinical signs.

(ii) Pilot clinical trial

A pilot clinical trial was conducted to evaluate the tolerability and potential efficacy of a formula combining *P. pentosaceus* CECT 8330 and *B. longum* CECT 7894. The study was designed as prospective, randomized, double-blind and placebo-controlled and involved a total of 8 centres in Spain. The study protocol was approved by the Ethics Committees of IDIAP Jordi Gol and the *Fundació Unió Catalana d'Hospitals*, and conducted in compliance with the Helsinki Declaration. A total of 31 infants were assessed for eligibility. Inclusion criteria were: infants 21-120 days old; birth weight ≥2.5 Kg; either breastfed or fed with infant formula; excessive crying and fussing according to the definition: "intense, persistent and inconsolable crying, problematic for the normal family unit functioning, which implies at least 60 minutes per day in 3 or more episodes in 3 or more days observed during at least 1 week, previously ruling out an organic etiology, such as intestinal intussusception or others". Exclusion criteria were: Pre-term infants (born before 37 weeks); chronic illness; history of gastrointestinal disorders; immunosuppressed infants; previous or expected surgical intervention; having taken probiotics or antibiotics one week before enrolment; infants whose parents or representatives were not able to appropriately follow the study requirements. Twenty met the selection criteria and were randomized to either receive probiotic or placebo using a computer-generated list (n=9 infants were assigned to placebo group and n=11 to probiotic group). The treatment consisted of daily administration for 14 days of 5 drops/day of a sunflower oil suspension providing $1x10^9$ CFU/day of *P. pentosaceus* CECT 8330 and *B. longum* CECT 7894 in 1:1 ratio. Placebo consisted in the same sunflower oil suspension without probiotic. Parents were asked to complete questionnaires recording the duration of crying and adverse effects and not to alter infants feeding during the study.

(iii) Statistics

Data analysis was performed with IBM® SPSS Statistic v20 and results expressed as means and standard errors. The mean reduction in daily crying time was calculated as the difference between the mean of the total number of minutes per day during the last 3 days of the study (days 12-14) and the mean of total number of minutes per day during the first 3 days (days 1-3). The mean reduction in the duration of each episode was calculated as the difference between the mean of the number of minutes each episode lasted during the last 3 days and the mean of the number of minutes each episode lasted during the first 3 days.The mean reduction in daily crying and minutes each episode lasted was compared between groups by means of unpaired t-test. Given the lack of similar studies using the same inclusion criteria and treatment time we were unable to make a pre-study sample size calculation, and the study sample was determined arbitrarily. However, sample size for future studies was calculated using PASS v13, with a minimum statistical power of 80% and 5% level of significance.

RESULTS

1. In vitro probiotic properties and toxicity test

The 16S rRNA sequence analysis confirmed a 99% and 100% identity for *Pediococcus pentosaceus* and *Bifidobacterium longum,* respectively. Both strains were deposited at the Spanish Collection of Type Cultures (CECT). *P. pentosaceus* CECT 8330 and *L. reuteri* DSM 17938 showed values of adherence to mucus of $1.40x10^6$ and $6.58x10^6$ CFU/cm, respectively, which were higher than those of *B. longum* CECT 7894 ($1.21x10^5$ CFU/cm). For adhesion to Caco-2 cells, the adherence value of *P. pentosaceus* CECT 8330 was $4.50x10^6$ CFU/cm, which compared well with those of *B. longum* CECT 7894 and *L. reuteri* DSM 17938 ($1.18x10^6$ and $1.01x10^6$ CFU/cm, respectively). The immunomodulatory capacity of the strains was studied by assessing their capacity to induce IL-10 production using a THP-1 cell line, commonly used for evaluating the anti-inflammatory potential of probiotics in macrophage-induced inflammation[21]. All three strains tested were able to induce the production of anti-inflammatory IL-10 (Table 1). The highest induction was observed after incubation in the presence of *P. pentosaceus* CECT 8330.

Table 1
Ability to induce IL-10 production in THP-1 macrophages

Conditions	24 h IL-10 (pg ml^{-1})
Negative control*	43.2
P. pentosaceus CECT 8330	140.2
B. longum CECT 7894	57.4
L. reuteri DSM 17938	122.2

Negative control corresponds to THP-1 macrophages incubated in the absence of bacterial strains.

The capacity of the LAB strains against potential intestinal pathogens is shown in Table 2. Both *P. pentosaceus* CECT 8330 and *B. longum* CECT 7894 were able to inhibit the growth of the whole spectrum of pathogens studied, although the activity of the latter was higher than the former.

Table 2
Antagonism against intestinal pathogens

Pathogen strain	*P. pentosaceus* CECT 8330	*B. longum* CECT 7894	*L. reuteri* DSM *17938*
Gram negative bacteria			
Escherichia coli ATCC 10538	3.0	> 6.0[a]	n.i*
Enterobacter aerogenes ATCC 13048	0.8	>6.0	0.8
Klebsiella oxytoca KT 801	5.4	>6.0	1.3
Bacteroides vulgatus ATCC 8482	2.1	>6.0	n.i
Gram positive bacteria			
Enterococcus faecalis ATCC 29212	3.5	>6.0	0.8
Clostridium difficile ATCC 9689	2.5	2.9	3.8

Results are expressed as growth inhibition zones (GI) calculated as GI= (IZD-CD)/2 where IZD and CD are the diameter of the inhibition zone and cylinder in millimeters, respectively.
[a] GI higher than 6 could not be measured due to halus overlapping.
**** n.i.; no inhibition observed.***

In contrast to *L. reuteri* DSM 17938, *P. pentosaceus* CECT 8330 and *B. longum* CECT 7894 did not produce gas. Additionally, neither *L. pentosaceus* CECT 8830 nor *B. longum* CECT 7894 were producers of histamine, putrescine, cadaverine, tyramine from histidine, ornithine, lysine or tyrosine. The production of D-lactate after 24h for both strains was low, being of 1.22 and 0.06 g/l, for *L. pentosaceus* CECT 8330 and *B. longum* CECT 7894, respectively. L-lactate production after 24h was 3.78 g/l for *L. pentosaceus* CECT 8330 and 3.82 g/l and for *B. longum* CECT 7894. Neither spontaneous mortality nor toxicity-related clinical signs were observed during the toxicity study. The behaviour of all animals was normal, and no differences were detected in body weight between control and *P. pentosaceus* CECT 8330. Moreover, no differences were observed between groups in the number of animals showing translocation of either LAB or enterobacteria to the liver (Table 3).

Table 3
Bacterial translocation in liver

		Enterobacteria*		Lactic acid bacteria**	
Group	**Sex**	***Animals showing translocation***	***Maximum translocation (cfu/10 mg)***	***Animals showing translocation***	***Maximum translocation (cfu/10 mg)***
Control	Males	0/4	0	2/4	1
	Females	1/4	1	3/4	7
P. pentosaceus CECT 8330	Males	0/4	0	1/4	3
	Females	1/4	3	1/4	20

** Bacteria plated on MacConkey growth medium.*
*** Bacteria plated on Man, Rogosa and Sharpes (MRS) growth medium containing 0.05% cysteine.*

2. Pilot clinical trial

P. pentosaceus CECT 8330 and *B. longum* CECT 7894 were combined in a single formula to evaluate its tolerability and efficacy in a pilot clinical trial. Baseline characteristics of participating infants can be found in Table 4. The median age for the total population was 66.1 days and the majority of the infants in both groups were boys (70%).

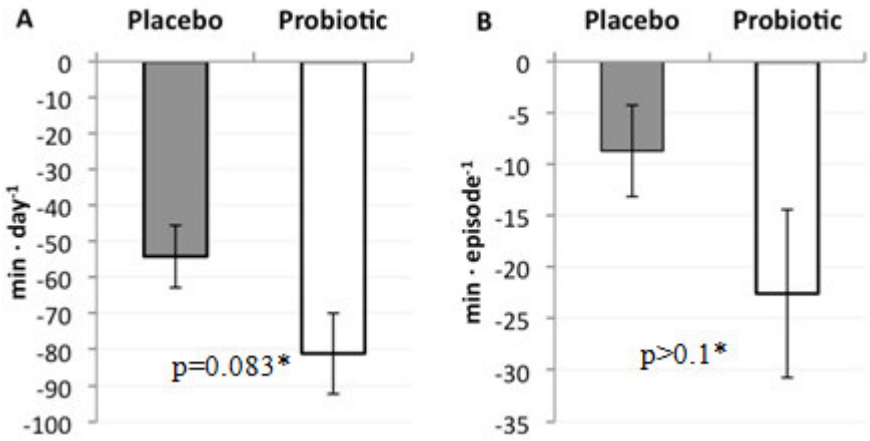
Table 4
Baseline Characteristics of Study Population

Variable	Probiotic group (n=11)	Placebo group (n=9)	Total (n=20)
Boys, %	60.0	75.0	67.7
Age at study entry, mean ± SD, days	53.5 ± 55.2	81.8 ± 43.6	66.1 ± 49.6
Birth weight, mean ± SD, g	3143.0 ± 259.1	3025.0 ± 314.6	3090.6 ± 273.0
Caesarean delivery, %	40.0	50.0	44.4
Baseline weight, mean ± SD, g	5188.8 ± 1584.7	5030.0 ± 1157.8	5109.4 ± 1287.6
Baseline length, mean ± SD, cm	55.3 ± 2.6	56.0 ± 5.4	55.6 ± 3.9

Both the probiotic and the placebo were generally well tolerated, and no adverse effects related to supplementation were observed. The mean crying time±SEM at the end of the treatment period was 38.9±15.0 and 20.4±5.6 minutes per day for the probiotic and placebo group, respectively. Crying time was reduced in each group ($p<0.05$) when compared to baseline. However, as shown in Figure 1, probiotic consumption tended to cause a greater reduction in daily crying time when compared to placebo (81.0 ± 11.2 vs 54.1 ± 8.6 reduction in minutes per day; P=0.083, statistical trend). A similar tendency was observed for the duration of

each episode, although results did not reach a statistical trend (22.5 ± 8.2 vs 8.7 ± 4.4 reduction in minutes each episode lasted; P>0.1). As this is a pilot study and sample size is small, we need to select an appropriate number of subjects for more consistent results. In order to guide future studies, we calculated the sample size based on the variance of the primary outcome. Therefore, a total of 18 infants for each arm of treatment are required to detect differences in the reduction of mean daily cry time using an unpaired t-test between two treatment groups, with an α level of 0.05 and a power of 80%.

Figure 1
Reduction in mean daily crying time and minutes each episode lasted



A) Reduction in mean daily crying time (total minutes cried per day). B) Reduction in mean duration of each episode (minutes each episode lasted). Results expressed as means ± SEM for n=9 in placebo group and n=11 in probiotic formula group. *Two-sample unpaired t-test

DISCUSSION

In the present study, we selected two LAB strains (*P. pentosaceus* CECT 8330 and *B. longum* CECT 7894) with probiotic properties of potential interest in the treatment of infant colic. Both selected strains were able to induce the production of anti-inflammatory IL-10, when we studied their immunomodulatory capacity. This is in line with other studies that have reported that *P. pentosaceus* strains are good modulators of cytokine production[22,23]. However, to the best of authors' knowledge, this is the first study showing the capacity of a *P. pentosaceus* strain to increase IL-10 production. The molecular basis of this cytokine modulation may be proposed to be ascribed to the interaction of the cell surface of probiotics with Toll-like receptors (TLR), mainly TLR-2 and TLR-4[21]. In addition, the capacity to inhibit the growth of intestinal pathogens was studied against potential intestinal pathogens. Both *P. pentosaceus* CECT 8330 and *B. longum* CECT 7894 were able to inhibit the growth of the whole spectrum of pathogens studied. This is consistent with the reported capacity of *Bifidobacterium* strains to modulate the intestinal microbiota[24] and the antimicrobial activity exhibited by other *P. pentosaceus* strains[25-27]. Remarkably, both strains showed good antagonistic activity against gas-producing enterobacteria belonging to *Escherichia* and *Klebsiella* genus as well as to *C. difficile*, known to be abnormally abundant in colicky infants[6,7]. On the other hand, *L. reuteri* DSM 17938 was unable to inhibit the growth of *E. coli*, which could partly explain its lack of effect on fecal microbial diversity in colicky infants, as reported in recent clinical trials[11,12]. Additionally, *B. longum* CECT 7893 also markedly inhibited the growth of *E. aerogenes* which has been reported to be significantly increased in infants with colic compared with control infants in the first 2 months[6]. In many susceptible infants, the excess of gas in the intestine

has been proposed as the cause of triggering colic[28]. In our study *P. pentosaceus* CECT 8330 and *B. longum* CECT 7894 did not produce gas, in contrast to *L. reuteri* DSM 17938, which is an interesting feature for administration to colicky infants. Although bifidobacteria have a long history of use as a probiotic in pediatrics[29], there is little evidence on their clinical benefits and tolerability in pediatric populations of *P. pentosaceus* strains. Therefore, neonatal rats were used due to the lower maturity of their digestive tract to provide a more stringent test for the safety of *P. pentosaceus* CECT 8330. Results of the toxicity assay showed that *P. pentosaceus* strain had no toxic effects on neonatal rats, which suggests beneficial data on the use of this strain for the treatment of infant colic. Due to the potential anti-inflammatory capacity of *P. pentosaceus* CECT 8330 and anti-microbial capacity of *B. longum* CECT 7894, it was decided to combine both strains in a single formula and evaluate its tolerability and efficacy in a pilot clinical trial. Infants who received probiotics tended to have a greater reduction in daily crying time from baseline compared with those given placebo. Since this is a pilot study, the sample size was limited, and larger studies are needed to confirm these results. In this way, we calculated the number of subjects required for a well-powered analysis. However, our study had strengths worth mentioning. For instance, the study included both breastfed and formula-fed infants, which is of relevant interest as a recent trial with a commercial probiotic failed to display any improvement in the formula-fed sub-population[11]. Moreover, participating infants were recruited based on a more realistic clinical definition of infant colic according to daily clinical practice. It is also worth mentioning that the treatment period (14 days) was shorter than that of many other clinical trials (21-28 days)[9-11]. A shorter treatment period can be considered of interest as some parents will be unwilling to wait several weeks before colic starts to subside, although it remains questionable whether the effect of the probiotic formula would be stronger after 21 or 28 days.

CONCLUSION

The results of this pilot study provide preliminary data suggesting that probiotic formula combining the novel *P. pentosaceus* CECT 8330 strain with *B. longum* CECT 7894 may greatly benefit infants with colic syndrome by reducing the daily crying time. Further larger clinical trials would enable the clinical relevance of these observations to be elucidated.

CONFLICT OF INTERESTS

JS, MCF, EL, JC are employees of AB-BIOTICS S.A., a Biotech Company interested in developing and licensing probiotic products.RGE focuses his professional activity on planning, implementing and evaluating pharmaceutical care activities in community pharmacy from the Council of Pharmacist in Catalonia. The other authors have neither economic nor employment relationship with any pharmaceutical company and declare that does not exist any conflict of interest. The Authors of this paper declares no conflict of interest.

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