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PROBIOTIC TREATMENT WITH AB-KOLICARE® CAUSES CHANGES IN THE MICROBIOTA WHICH CORRELATE WITH A REDUCTION IN CRYING TIME

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ABSTRACT

Growing interest is being placed on the role of intestinal microbiota as several studies have correlated alterations in diversity and abundance of relevant bacterial subpopulations with conditions without apparent etiology, such as infant colic. In this work, we study the changes in the microbiota of colicky infants induced by the treatment with AB-Kolicare, a probiotic formula comprised by *Pediococcus pentosaceus* CECT 8330 and *Bifidobacterium longum* CECT 7894. A significant increase in the microbiota diversity was found in infants treated with AB-Kolicare when compared to placebo, which correlated with a reduction in the total crying time. In addition, relative abundance of several phyla and genera which are described to be increased in colic was reduced after treatment, and a significant increase in the rate of subpopulations which protect against colic was also observed in comparison to placebo.

KEYWORDS: **Infant colic, excessive crying syndrome, intestinal microbiota, probiotic, diversity.**

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INTRODUCTION

The incidence of excessive crying syndrome, also commonly referred as infant colic, varies from 5% to 20% (or even 40%, depending on the criteria employed) and it is considered the cause of 10-20% of all pediatrician visits in the first 4 months of life.[1] Infantile colic is a behavioral syndrome which typically occurs in apparently healthy infants. Although high number of definitions of colic exist, no consensus has been reached yet for excessive crying.[2] Infant colic was defined in 1954 as an excess of crying or paroxystic irritability without an apparent cause, for more than three hours a day, occurring more than three days a week in three consecutive weeks.[3] Later on, the Rome Coordinating Committee III defined that these same crying episodes more than three hours in three days of a single week were enough to establish an infant colic diagnostics.[4] However, different concerns have arisen regarding these definitions as, in fact, in the daily practice of primary healthcare centers and hospitals, there is a noticeable population of infants that, albeit does not perfectly fit these criteria, show an abnormal and unexplained crying. Nowadays, the most frequent clinical practice is to consider the excess of paroxystic crying episodes without an apparent cause as infant colic, especially when altering significantly and repetitively the quality of life of the parents. Infant colic is characterized by excessive paroxysmal crying that it is most likely to occur in the late afternoon or evening. The crying usually begins suddenly, for no clear reason, is intense and often high pitched.[5] Moreover, 'colicky infants' are usually very unsettled, fussy or irritable without definite explanation, or may show signs of pain (e.g. drawing up knees or arching the back).[6] Colic is often a serious problem for parents as excessive crying leads to parental exhaustion and has many deleterious consequences including difficulties with concentration, loss of patience, frustration, feelings of incompetence, fear of harming the child, early cessation of breastfeeding and reduction of face-to-face interaction with their child.[6,7] To date there is no clear knowledge of colic's etiology, but a variety of causes have been suggested, including abnormal gastrointestinal function, immaturity of the gut, spastic colon, and gas accumulation or allergic problems.[8] Correlation between dysbiosis and gastrointestinal symptoms has been widely established by the assessment and understanding of metagenomic data in pediatric patients.[9] Several studies correlate alterations in gut microflora and digestive discomfort with infant crying syndrome and infant colic,[10–15] suggesting that differences in the gut microflora could play an important role in the pathogenesis of colics.[10,11,13,16] Moreover, a study based on microarray revealed that infants with colic showed lower microbiota diversity and stability than control infants in the first weeks of life.[11] Lower counts of intestinal lactobacilli were observed in colicky infants in comparison with healthy infants.[13,17] Evidence suggests that high prevalence of *Bifidobacterium* and *Lactobacillus* in the microbiota of infants may protect against crying and fussing.[18] In general, alterations comprise the reduction of *Actinobacteria Bifidobacterium* and *Firmicutes Lactobacilli* species rates, and the overgrowth of *Chlostridium, Bacteroides, Klebsiella, Enterobacter, Staphylococcus* and other gram negative anaerobic species such as *Escherichia* or *Shigella.*[10–12,14,15,20] *Proteobacteria* are also increased, including species producing gas and inflammation.[10] This bacterial unbalance leads to changes in the metabolome of gut microbiota, the ensemble of gut microbiota byproducts which have a positive impact on human health. Bacterial metabolome changes may affect to the infant carbohydrate and fatty acids metabolism and lead to a deregulation of intestinal motor function and to an increase in gas production and inflammation which causes pain and general digestive discomfort.[13] Some studies have shown that administration of *Lactobacillus* strains is well tolerated and improves symptoms of infantile colic in breastfed infants.[21–23] Putative mechanism of action include improvement in gut function and motility, as well as a possible effect on visceral pain. In addition, probiotics may contribute to the improvement of symptomatology by modulating immune response and correct intestinal dysbiosis.[24] In this randomized, double-blind and placebo-controlled pilot clinical trial, a mixture of two lactic acid bacteria - *Pediococcus pentosaceus* CECT8330 and *Bifidobacterium longum* CECT7894 was administered to 10 infants who exhibited a daily crying duration of 55-155 min/day (mean 104 min/day). Fecal samples obtained before and after treatment with placebo or probiotic were analyzed by metagenomic methods with the objective to observe a recovery of the microbiota balance and a correlation with a decrease in the number of crying episodes and duration.

MATERIALS AND METHODS

Bacterial strains

Perdiococcus pentosaceus CECT 8330 and *Bifidobacterium longum* CECT 7894 lactic acid strains with QPS status (Qualified Presumption of Safety by the EFSA (EFSA-Q-2005-293). These strains were previously isolated and characterized for their properties and mechanism of action to treat infant colic.

Pilot Clinical trial: study design

Fecal samples from a randomized, double-blind and placebo-controlled pilot clinical trial were analyzed by means of metagenomics to evaluate the changes in the microbiota caused by the probiotic formula combining *P. pentosaceus* CECT 8330 and *B. longum* CECT 7894. The study was designed as a case-control clinical trial, involving a single participating center in Spain (Clínica Terres de l'Ebre, Tortosa, Spain). The primary endpoint of the study was to measure the correlation between the time of crying of the colicky infants and the changes observed in the microbiota before and after treatment with AB-Kolicare or a placebo. The study protocol was approved by the Ethical Committees from IDIAP Jordi Gol (Barcelona, Spain) and from Fundació Unió Catalana d'Hospitals (Barcelona, Spain) in compliance with the Helsinki Declaration. All infants' parents gave written informed consent to participate. Eleven healthy term infants of both sexes meeting all of the following inclusion criteria were recruited: from 21 to 120 days old; minimum birth weight of 2.5 Kg; either breastfed or feed with infant formula (hydrolyzed or initiation 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 50 minutes of crying per day in 3 or more days observed during at least 1 week, previously ruling out an organic etiology, like intestinal intussusception or others". Exclusion criteria were: Pre-term infants (born before 37 weeks); chronic illness; history of gastrointestinal disorders (not related to colic); immunosuppressed infants; previous or expected surgical intervention; having taken probiotics or antibiotics one week before the enrollment; infants whose parents or representatives were not able to appropriately follow the study requirements. At a first stage, the eleven colicky infants recruited were randomly assigned to the placebo (n=5) or the treatment group (n=6). Samples from one of the subjects in the placebo group did not yield enough DNA for amplification and were discarded accordingly, reducing the number of subjects in the placebo group to n = 4 and the total N to 10 subjects. Subjects were randomly assigned to either probiotic treatment group (n =6) or placebo group (n=4) using a computer-generated list. The treatment consisted in a sunflower oily suspension containing *P. pentosaceus* CECT 8330 and *B. longum* CECT 7894 (dose of 1E+09 to 1E+10 CFUs in a ratio of 1:1 of each probiotic strain during the study), administered orally. Placebo consisted in the same oily suspension without probiotic. Compositions were administered 30 minutes before feeding (5 drops/day) for 14 days. During the study, parents were asked to fill questionnaires to record the duration of crying. Fecal samples before and after treatment were collected by the parents at home and stored at -20ºC until DNA extraction.

DNA extraction, PCR amplification and massive sequencing

Bacterial DNA was extracted from the samples using an automatized MagNa Pure extractor (Roche) and the MagNa Pure LC DNA Isolation Kit III (Bacteria, Fungi)(Roche) under manufacturer's conditions. DNA samples were eluted in 100 µl and stored at -20°C until further processing. 16S rDNA gene amplicons were amplified following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Part # 15044223 Rev. A). The gene-specific sequences used in this protocol target the 16S rDNA gene V3 and V4 region. Illumina adapter overhang nucleotide sequences are added to the gene-specific sequences. The full length primer sequences which were selected from the procedure described in Klindworth *et al*.,[25] using standard IUPAC nucleotide nomenclature, to follow the protocol targeting this region are:

16S rDNA gene Amplicon PCR Forward Primer =
5'-
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC
TACGGGNGGCWGCAG-3'
16S rDNA gene Amplicon PCR Reverse Primer =
5'-
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG
ACTACHVGGGTATCTAATCC-3'

Microbial Genomic DNA (5 ng/µl in 10 mM Tris pH 8.5) was used to initiate the protocol. After 16S rDNA gene amplification, the mutiplexing step was performed using Nextera XT Index Kit (FC-131-1096). 1 µl of the PCR product was run on a Bioanalyzer DNA 1000 chip to verify the size, the expected size on a Bioanalyzer trace is ~550 bp. After size verification, the libraries were sequenced using a 2x300pb paired-end run (MiSeq Reagent kit v3 (MS-102-3001)) on a MiSeq Sequencer according to manufacturer's instructions (Illumina).

Quality control, OTU assignment, diversity, composition and functional analyses

Quality assessment was performed by the use of prinseq-lite program applying a minimum length of 50 bp. a trim_qual_right of 30, a mean trim_qual_type and a trim_qual_window of 10 bp. R1 and R2 from Illumina sequencing where joined using *fastq-join* from *ea-tools* suite.[26] Regarding the bioinformatics analysis, data were obtained using an *ad-hoc* pipeline written in RStatistics environment,[27] making use of several Open Source libraries such as *gdata*, *vegan*, etc. Taxonomic affiliations have been assigned using the *RDP_classifier* from the Ribosomal Database Project.[28] Reads with RDP score value below 0.8 were assigned to the upper taxonomic rank leaving last rank as unidentified (e.g. *Firmicutes, Bacillales, Bacillaceae*, Unidentified *Bacillaceae*).Alfa diversity (number of different species per sample),was characterized for every sample at *Phylum*and *Genus* taxonomy ranks levels. Shannon, Simpson and inverse Simpson were calculated.

Statistical analysis

Data analysis was carried out within the R statistics environment.[27]The differences in proportion of the different taxa identified between groups and within the same group are analyzed with Students' *t*-test for unpaired samples and paired samples, respectively. Correlation was measured using Pearson's R value.Atwo-sided adjusted value of $p < 0.05$ was considered statistically significant.

RESULTS

Colicky infants' characteristics

The average initial crying time was found to be 103.77 ± 32.73 minutes, being 93.89 ± 34.16 in the probiotic group and 118.58 ± 27.97 in the placebo group.

Table 1
Demographic data of infants at baseline

Variable	Placebo (n=4)	Probiotic (n=6)	Total (n=10)
Male, %	75.00	50.00	60.00
Age, average ± SD, days	71.85 ± 03.62	55.50 ± 35.62	62,04 ± 21,62
Birth weight, average ± SD, gr	3020.00 ± 114.23	3201.00 ± 59.10	3128.60 ± 54.70
Caesarean-born, %	50.00	50.00	50.00
Initial weight, average ± SD, gr	5130.00 ± 1157.80	5088.80 ± 1584.70	5105.33 ± 1413.94

Initial height, average ± SD, cm	56.10 ± 5.30	55.70 ± 3.60	55.86 ± 4.30
Initial crying time ± SD, min	118.58 ± 27.97	93.89 ± 34.16	103.77 ± 32.73

Both the probiotic and the placebo were generally well tolerated, and no adverse effects related to supplementation were observed.

Reduction in crying time

Table 2 summarizes the reduction in crying time observed after 2 weeks of treatment with AB-Kolicare or placebo. Basal crying time in the probiotic group was slightly lower, even if differences between the placebo and the probiotic group at time 0 were not significant. Treatment with AB-Kolicare achieved a reduction of 69.11 ± 13.80% (*t*-test T0 vs. T14, $p = 0.002$), while the placebo group exhibited a decrease of 43.49 ± 8.08% (*t*-test T0 vs. T14, $p = 0.014$).

Table 2
Reduction in crying time after treatment

Crying time	Placebo (n = 4)	Probiotic (n = 6)
At time = 0 days, min ± SD	118.58 ± 27.97	93.89 ± 34.16
At time = 14 days, min ± SD	65.58 ± 8.71	37.00 ± 50.05
Reduction, % ± SE	43.49 ± 8.08%	69.11 ± 13.80%

Phyla relative abundance before and after treatment with placebo and probiotic

Figure 1A shows the relative abundance at the phylum level of initial samples at time = 0 and samples after treatment (t = 14 days), either for the placebo or the probiotic group. Some differences at baseline can be observed between the placebo and the probiotic group, especially in the relative abundance of the *Firmicutes* phylum, increased by two-fold in the placebo group. After the probiotic treatment, clear changes in the Phyla relative abundance can be observed: *Actinobacteria* were found to be increased (15.67±14.75%), while this increase was slighter in the placebo group (5.23±9.29%). *Proteobacteria* were decreased in the probiotic group after treatment (-8.59±5.75%) while in contrast an increase (10.86±10.31%) is observed in the placebo group after 14 days. Finally, a reduction in *Firmicutes* can be observed after treatment in both the probiotic and the placebo group, but this decrease is milder in the probiotic group (Placebo -16.41±5.34%, Probiotic -8.76±13.46%). The changes of these Phyla over time are depicted in Figure 1B.

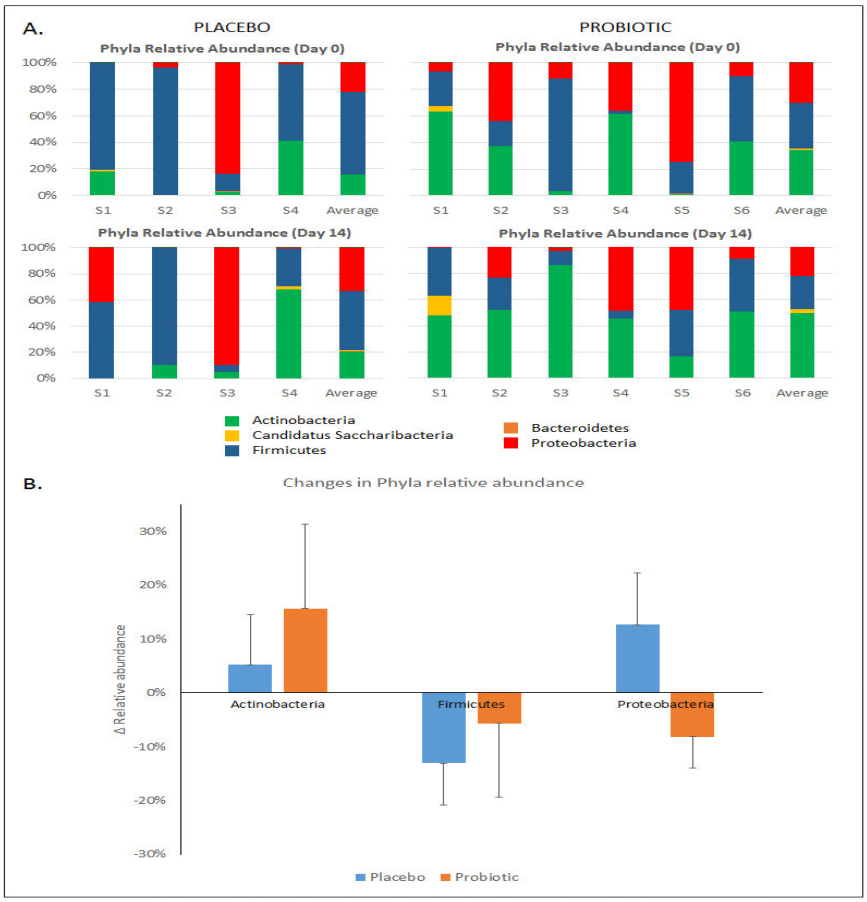

Figure 1. A. Phylum relative abundance in the placebo and probiotic groups at time = 0 and time = 14 days. **B.** Changes in Phylum relative abundance after treatment with placebo or AB-Kolicare.

Figure 1
Changes in relative abundance at the Phylum level after treatment with placebo or AB-Kolicare.

Diversity analysis before and after treatment with placebo and probiotic

Diversity among samples at phylum level was compared using the Shannon α-diversity index and the inverse Simpson α-diversity index, in order to confirm that the results obtained by these two different and standard indexes for diversity analysis were consistent. Changes in α-diversity are represented in Figure 2. An increase was observed when comparing samples before and after treatment, in both the placebo and the probiotic groups and for both diversity indexes. However, the increase in diversity observed at baseline was not statistically significant, while the inter-group difference between placebo and probiotic in Shannon and inverse Simpson's indexes was significant after treatment (p = 0.036 and p = 0.042, respectively). Of note, this larger increase in the probiotic group occurred despite starting from values which were already higher at baseline than placebo, although the pairwise difference did not reach statistical significance.

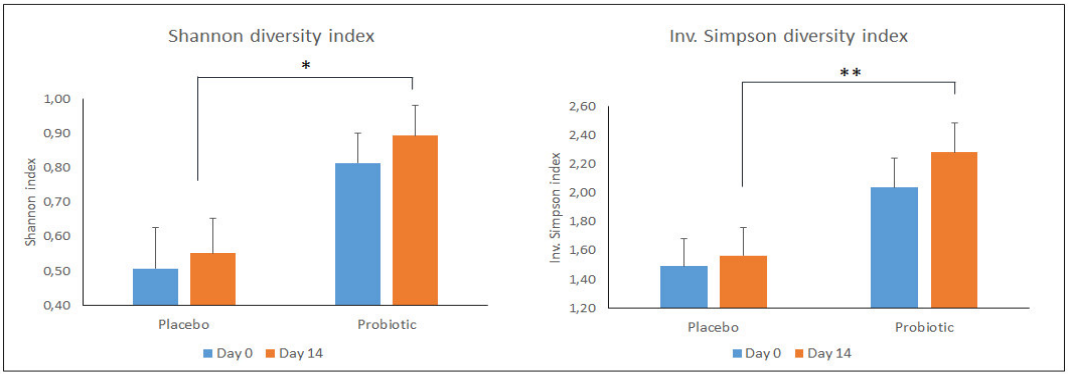

Figure 2. A. Shannon diversity index increment after treatment with probiotic or placebo. Differences between the placebo and the probiotic groups were significant after 14 days of treatment (*p = 0.036). **B.** Inverse Simpson diversity index increment after treatment with probiotic or placebo. Differences between the placebo and the probiotic groups were significant after 14 days of treatment (**p = 0.042). Differences between placebo and probiotic at time 0 were not significant.

Figure 2
Changes in diversity after treatment with AB-Kolicare.

Correlation of phyla diversity with crying time

The reported increase in Phyla diversity correlated with a reduction in total crying time by Pearson's correlation. The correlations were calculated taking into account all the available data for diversity and crying time, including the placebo and the probiotic samples, and are depicted in figure 3. Correlation between the increase in Phyla diversity measured by the Shannon index and the reduction of the total crying time was significant (r=0.80, p = 0.006) and is represented in figure 3A. Moreover, the correlation of the increase in the 1 - Simpson index with the reduction of the total crying time was also significant (r=0.66, p = 0.038) and is observed in Figure 3B.

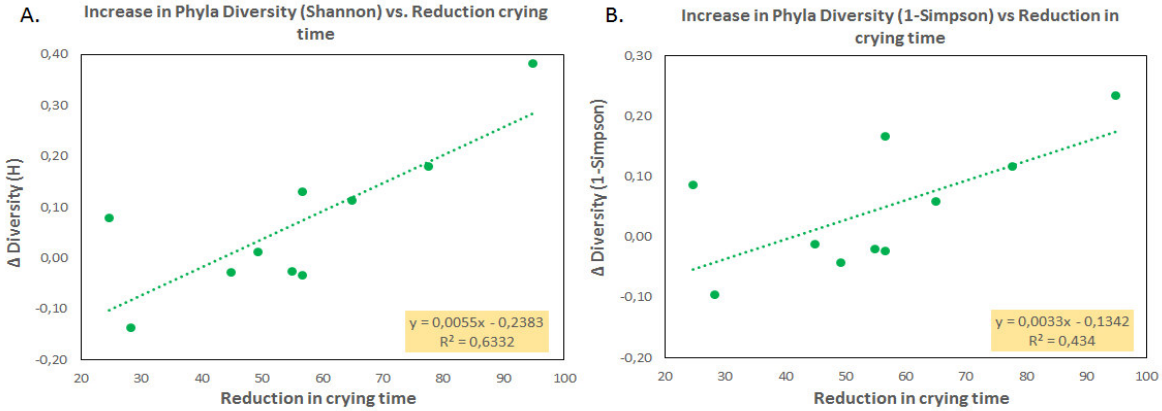

Figure 3. Pearson correlation between the increase in α-diversity indexes and the reduction in total crying time. **A.** Pearson correlation with Shannon index (p = 0.006) **B.** Pearson correlation with 1 – Simpson index (p = 0.038).

Figure 3
The increase in Phyla diversity correlated with a reduction in total crying time

Changes in Genera relative abundance after AB-Kolicare treatment.

Several changes could be observed at the Genus level after treatment with AB-Kolicare when compared to placebo. Changes concerning genera correlated with infant colic are reported in Figure 4. A decrease in genera positively correlated with the presence of colic in infants[11] was detected after treatment with the probiotic

formula, while these same genera were slightly or clearly increased in all cases after treatment with placebo. These genera include *Escherichia/Shigella, Klebsiella, Enterobacter* and *Staphylococcus*, and in addition, the reduction in *Staphylococcus* abundance after treatment correlated with a decrease in the crying time (p = 0.019). A marginally significant increase in *Bifidobacterium* was observed after treatment with the probiotic formula when compared to placebo (p = 0.056). This increase was found to be relevant as *Bifidobacterium* have been described in many works as a protective subpopulation against infant colic.[11,18,29]

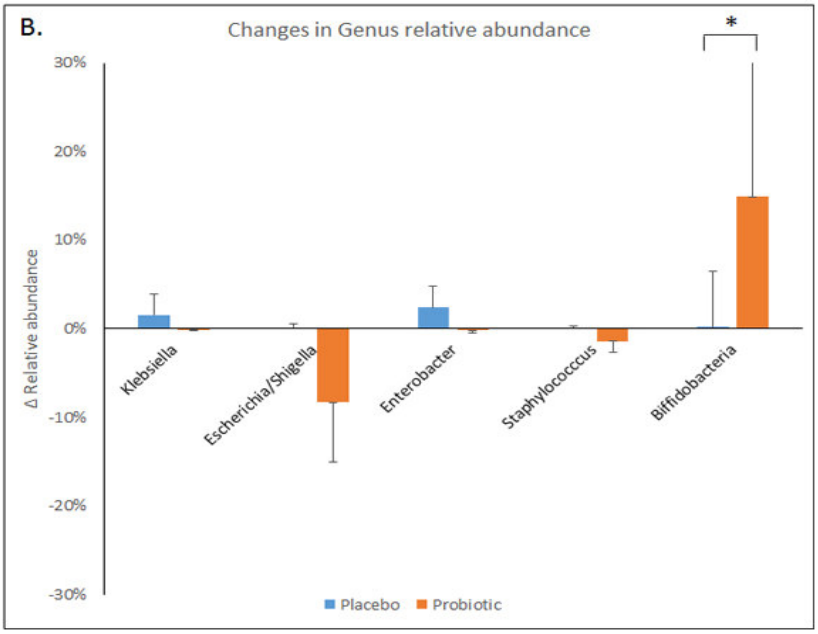

Figure 4. Changes in genus relative abundance. *Significant differences between placebo and probiotic groups were observed after treatment. Baseline: $p = 0.121$ After 14 days of treatment: $p = 0.028$.

Figure 4
Changes in relative abundances at genus level

DISCUSSION

Alterations in colicky infants' microbiota with respect to babies who do not exhibit an erratic crying behaviour have been widely studied.[10,11,13,14,18,29–31] Putting together all the results reported in these works, a microbiota profile of colicky infants can be established in which a reduction in α-diversity[11,31] and in the relative abundance of several subpopulations such as *Bacteroides,*[29] *Firmicutes,*[13,14,16,18,29] or *Actinobacteria*,[18,29] and an overgrowth of *Proteobacteria*[11,29] can be linked to a colic pattern. Within these Phyla alterations, several changes in the abundance of particularly relevant genera have been described. As an example, decreases in *Lactobacillus* sp. or *Bifidobacterium* sp. are widely accepted as alterations with causal relevance in infant colic etiology. A possible mechanistic hypothesis for the protective effect against infant colic might be related with the fact that mucosal *Lactobacillus* are able to induce the expression of anti-inflammatory genes,[32] but this mechanism still needs to be validated in infant colic. Recent observations indicate that increases in several *Proteobacteria* genera known as potential pathogens which cause inflammation, such as *Enterobacter, Escherichia, Shigella* or *Klebsiella,* are positively associated with infant colic.[11,30,31,33] Some of these anaerobic genera are known to produce gas as a result of their metabolism,[34] which could be related with bloating and pain in infants exhibiting an excessive crying syndrome. The improvement in crying symptoms achieved by AB-Kolicare can be attributed to a correction of the dysbiotic microbiota found at baseline, even if in some cases the effects were not significant due to statistical limitations. Changes in the relative abundances of several subpopulations after treatment with AB-Kolicare® are consistent with the previous findings described above. We observed an increase in *Actinobacteria* due to a rise in *Bifidobacterium* (14.85 points), and a decrease in *Proteobacteria,* related, between others, with the decrease in the *Escherichia/Shigella* subpopulation, representing a reduction of 8.34 points. Remarkably, the increase in *Actinobacteria* was higher in the probiotic group when compared to placebo, and in *Proteobacteria* the effect is only observed in the probiotic group, being this subpopulation increased over time in the placebo group. This rise over time in the placebo group puts into higher value the reduction in colic-associated *Proteobacteria* achieved by AB-Kolicare treatment, as a difference of 19.45 points is observed at day 14 between the probiotic and the placebo group. In contrast, we did not detect an increase in the relative abundance of *Firmicutes*, as it could be expected from previous results. However, the reduction in *Firmicutes* was milder in the probiotic group when compared to placebo. We speculate that this limited and unexpected reduction in *Firmicutes* after treatment with AB-Kolicare might be due to the lower abundance of some genera belonging to this Phylum, such as *Staphylococcus* or *Streptococcus*, which reduced the relative abundance of *Firmicutes* in 1. 39

and 18.12 points, respectively. Interestingly, *Staphylococcus* has been previously reported to be a genus positively correlated with the presence of colic.[11] In our study, the observed reduction in *Staphylococcus* after treatment correlated with a decrease in the total crying time (p = 0.019). In addition, this effect was not observed in the placebo group. For this reason, even if a reduction in *Firmicutes* was considered a surprising finding at a first stage, the subsequent analysis at the genus level explained the results and confirmed that the treatment with AB-Kolicare was able to alter the microbiota in a way that decreased several subpopulations associated with colic and increased the rate of bacteria with a protective affect against colic. Finally, an increase in microbiota α-diversity analysed by both Shannon and inverse Simpson diversity indexes was observed after treatment with both placebo and AB-Kolicare, while differences at baseline were not significant. Notably, differences between the placebo and the probiotic groups in diversity indexes Shannon and inv. Simpson were significant after 14 days of treatment (p = 0.036 and p = 0.042, respectively). In line with previous reports,[11,31] the increase in α-diversity correlated with a reduction in the total crying time, confirming that AB-Kolicare was able to improve the microbiota profile of colicky infants and their symptoms and well-being, and as a consequence, the parents' quality of life. This study represents a pilot exploratory clinical trial with a total of 10 subjects. Even assuming the statistical limitations of a small sample size, the degree of improvement in the microbiota profile of colicky infants was enough to detect changes in the probiotic group which were not seen in the placebo group, and this improvement correlated with a reduction in the total crying time. A reduction of almost 70% in crying time was achieved by AB-Kolicare®, clearly superior to the decrease observed in the placebo group (around 43%). The statistical significance of the reduction in crying time for the probiotic group was higher than that observed for the placebo group ($p < 0.005$ vs. $p < 0.05$ in the placebo group). The reduction of crying time in the placebo group can be explained by the important placebo effect that has been observed in previous studies[22,35] and in particular, has been repeatedly observed in infant colic.[36] In addition, the changes in the microbiota profile, which represent the main mechanism of action of AB-Kolicare, are only observed after treatment with the probiotic. This study sheds light on the mechanisms of AB-Kolicare for improving daily crying, related to alterations in microbiota which are in accordance with previously reported observations. Future studies with larger number of subjects are currently underway to confirm these findings.

CONCLUSION

Metagenomic analysis of stool samples from infants suffering from excessive crying syndrome treated with AB-Kolicare provided a deeper comprehension on the effects of the probiotic formula in balancing dysbiosis and reducing the gastrointestinal symptoms of this affection. The metagenomic analysis has proven that the recovery of the microbiota balance provided by AB-Kolicare is correlated with a higher reduction of the total crying time in infants with colic when compared to placebo. This decrease in crying and fussing implies an improvement in colic symptoms, impacting positively in the infant comfort and consequently in the quality of life of the parents.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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