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Use of a probiotic mixture containing *Bifidobacterium animalis* subsp. *lactis* BB12 and *Enterococcus faecium* L3 in atopic children

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Running header: BB12 and L3 bacterial strains in atopic children

Abstract

BACKGROUND: Imbalance of the human gut microbiota in childhood, mainly due to low gut biodiversity and a low bifidobacterial load, has been suggested as a risk factor for atopy. Administration of *Enterococcus faecium* L3 in infants has been shown to increase the gut bifidobacterial count. The aim was to verify if a mixture of *Bifidobacterium animalis* subsp. *lactis* BB12 and *E. faecium* L3 could reduce the signs, symptoms and need for drugs in atopic children.

METHODS: We retrospectively analyzed, and compared with controls, clinical outcomes following use of BB12 and L3 strains when administered 3 months before or during the development of signs and symptoms of atopy.

RESULTS: When administered in the 3 months before the development of atopy, the BB12 and L3 strains significantly reduced ($p<0.001$) rhinitis, watery eyes and cough/bronchospasm. However, reduced efficacy was observed when the mixture was given during the 3 months of atopy. The mixture of strains also significantly reduced the use of oral anti-histamines, inhaled corticosteroids (in the same children in two different years) and oral corticosteroids (in different children in the same year).

CONCLUSIONS: When administered as a prophylactic, the mixture of BB12 and L3 (iNatal Ped®) statistically decreases the signs and symptoms of atopy and reduces the use of drugs. Administration of the same probiotics as treatment after the appearance of atopy is less effective.

Key words: Allergy, asthma, iNatal Ped®, atopic treatments, rhinitis, watery eyes, cough, bronchospasm

Introduction

The human gut microbiota consists of 500–1000 distinct bacterial species with millions of active genes capable of influencing the host immune system and the mechanisms of atopy.[1] Most of these bacterial species belong to five phyla: Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia, with Bacteroidetes and Firmicutes constituting more that 95% of all gut bacteria.[2] Imbalance of the human gut microbiota in early childhood has been suggested as a risk factor for atopy.[3] *Clostridium difficile* colonization early after birth, for instance, was associated with gasping during the first 6–7 years of life and with asthma at age 6–7 years.[4] Moreover, children developing allergy in the first decade of life had less gut bacterial diversity in the first month after birth compared with non-allergic children,[5] as well as a lower content of *Bifidobacterium* and *Enterococcus* species.[6] Lower relative abundance of bacteria such as *Bifidobacterium*, *Akkermansia* and *Faecalibacterium* in children has been implicated in childhood atopy and asthma[7] and lower content in *Bifidobacterium* has been observed in some cases of adult asthma.[8] A lot of evidence also supports the immunomodulatory effects of some bifidobacterial strains, such as *Bifidobacterium animalis* and *Bifidobacterium longum*. These strains seem to reduce the Th2 immune response and antagonize the pro-inflammatory LPS-driven cascades.[9] *Enterococcus faecium* L3, a probiotic strain known for its ability to release the bacteriocins enterocin A and enterocin B which target gut and vaginal pathogens, when administered to newborn infants preserves and increases the growth of endogenous bifidobacterial species, in addition to reducing the number of opportunistic microorganisms.[10] We therefore retrospectively evaluated whether the administration to atopic children of a mixture of the two well-documented probiotic strains *B. animalis* subsp. *lactis* BB12 and *E. faecium* L3 was able to reduce the signs and symptoms of atopy and/or to reduce the use of drugs administered to control asthma and allergy.

Materials and Methods

Product

The probiotic mixture of *B. animalis* subsp. *lactis* BB12 (BB12) and *E. faecium* L3 (L3) was manufactured by Farmaceutici Procemsa (Nichelino, Turin, Italy) as single dose

sachets. The product was notified to the Italian Ministry of Health on June 16, 2016 as iNatal Ped® by Omeopiacenza (Pontenure, PC, Italy) according to the provisions of law No. 169 of 2004 (notification number: 87470). The preparation used in the study contained not less than 2 billion CFU/sachet of each strain (total dose: not less than 4 billion CFU/sachet) as declared in the expiry date.

Clinical trial

Our retrospective analysis was conducted on 89 (54 male and 35 female) children with atopy living in the Milan area of Italy (see Figure 1 for the study scheme). Some of the children (n=46) were treated with the probiotic mixture, while the remaining 43 served as untreated controls. Children were randomized to the treated or control group depending on whether a flipped coin landed heads (treated group) or tails (control group). In the prophylactic arm of the study, children were treated between January and March (2017) in the 3 months before the 'allergic' period (April–June, 2017), while in the treatment arm, children were treated during the allergic 3 months (April–June, 2017). As one of the researchers (MLD) joined the study in the middle of the prophylaxis 3 months, children enrolled by this physician (n=22) were treated during the allergic 3 months. We compared the results in the children who received the mixture (n=46) with the same children the year before (untreated 2016) and with another group of untreated children (n=43) enrolled as a control group in 2017 (untreated 2017). During the allergic 3 months, all children (n=89) were administered physician-prescribed conventional pediatric drugs to control atopy as required. The retrospective analysis was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committee in Milan (Italy). The parents of all study participants were informed of the study methods and signed the appropriate consent and privacy policy documents.

Inclusion and exclusion criteria

We included children living in the northern part of Milan, who were 2–14 years of age and attending one of our pediatric outpatient clinics with a diagnosis of atopy based on a skin prick test, radioallergosorbent (RAST) test and clinical examination. The children enrolled were all characterized by atopy versus inhaled allergens occurring in the 3 months April–June. We excluded children who were not immunocompetent, had cardiomyopathy, intrinsic asthma or wheezing due to infective etiology, or current or previous use of desensitizing vaccines.

Treatments

The parents were asked not to give their children any other probiotics, including yogurt and dead bacterial cells (tyndallizated bacteria, bacterial lysate, post-biotics) for the duration of the study. In case of antibiotic use, parents were told to stop probiotic administration and to resume it after the last antibiotic dose had been given. Acetaminophen or ibuprofen could be used to treat pain, in which case probiotic use could be continued. Parents were asked not to administer the probiotics when the child was hungry or had consumed a large meal so that the probiotics would survive better to colonize the gut. All children were treated with conventional anti-atopic drugs during the 3 months April–June in 2017 according to their prescriptions (which were similar to those for the same 3 months in 2016). Any conditions or diseases compatible with the aim the study were managed in accordance the Italian pediatric guidelines. There were no cases of diseases incompatible with our study and all enrolled children completed the study.

Study aims

The primary endpoints of the study were tolerability, side effects and compliance with the probiotic treatment, and development of signs and symptoms of atopy (rhinitis, watery eyes, cough and bronchospasm). Symptoms were evaluated according to a visual analogue scale as follows: 0 = no signs; 1 = mild signs; 2 = moderate signs; 3 = severe signs. The secondary endpoint was the need for and use of conventional drugs such as oral and ophthalmic anti-histamines, oral and inhaled corticosteroids, anti-leukotrienes and adrenergic β2 agonists. Topical corticosteroids were not used since no child had a diagnosis of dermal atopy.

Statistical analysis

Aiming for a significance level of 10% and a power of 90%, with an effect size of at least 0.55, we determined that a sample size of at least 32 subjects was required in order that the 2017 prophylactic group could be compared with their 2016 untreated status. Aiming for a significance level of 10% and a power of 90%, with an effect size of 0.8, we determined that at least 60 subjects were required, 30 for each arm, in order that the 2017 treated and 2017 untreated group could be compared. Therefore, at least 60 subjects, 30 for each arm, needed to be enrolled.

The non-parametric Wilcoxon signed-rank test was used to compare the treated 2017 and untreated 2016 groups. The non-parametric Wilcoxon rank-sum test was used to compare the treated 2017 and untreated 2017 groups. Results are presented as the mean and standard deviation or the odds ratio, with 95% confidence intervals.

To determine the odds ratios, we created a new variable for the presence, or not, of symptoms or the use, or not, of conventional drugs during the observation period. Therefore, the variable did not indicate the number of times a symptom occurred or the number of days a drug was used. JMP 10 for Mac OS X was used for statistical analysis and statistical significance was set at 95%.

Results

Our aim was to determine if treatment with a mixture of *B. animalis* subsp. *lactis* BB12 and *E. faecium* L3 (BB12 and L3) in addition to being well tolerated, lacking side effects and having good compliance, was able to reduce the signs, symptoms and need for drug use in atopic children. As shown in Tables 1 and 4, children enrolled in the prophylactic and treatment arms of the study did not show any differences in age or sex. Similarly, there were no significant differences in birthweight, type of delivery (vaginal or cesarean), breastfeeding (or not), ethnic group (Caucasian or not), or attendance at a nursery, toy library or kindergarten (data not shown) between the groups. The BB12 and L3 mixture was very well tolerated by all children and did not cause any side effects. Compliance was also good and no child withdrew from the study (data not shown).

Children in the prophylactic arm showed a highly significant ($p<0.001$) reduction in signs and symptoms of atopy (Tables 2 and 3). Rhinitis, watery eyes and cough/bronchospasm were reduced by approximately 50% in children treated in 2017 as compared with the same children untreated in 2016 and as compared with the other children untreated in the same 3 months of 2017.

In contrast, children in the treatment arm of the study only showed a tendency towards a reduction in signs and symptoms (Tables 5 and 6). When different children treated or not in the same year (2017) were compared, a significant ($p<0.05$) reduction in rhinitis and watery eyes was seen; when the same children from two different years (2017 versus 2016) were compared, only cough/bronchospasm was significantly reduced ($p<0.05$).

Analysis of signs, symptoms and drug use in children administered the probiotic mixture (both prophylactically and not) in 2017 compared with untreated children in the same year, showed a significant association between the use of BB12 and L3 and a reduction in rhinitis, watery eyes and cough/bronchospasm (O.R.=0.2) and a decrease in the number of days of treatment with conventional drugs such as oral corticosteroids (O.R.=0.5) (Table 7). Comparison of the findings in the same group of children untreated in 2016 but treated in 2017, showed a significant association between the use of probiotics and a reduction in cough/bronchospasm (O.R.=0.4) and a decrease in the number of days of treatment with conventional drugs such as oral anti-histamines, inhaled corticosteroids and adrenergic β2 agonists (O.R.=0.2, O.R.=0.3 and O.R.=0.4, respectively) (Table 8).

Discussion

B. animalis subsp*. lactis* BB12 (BB12) is a well-documented probiotic. It has been described in more than 300 scientific papers including over 130 clinical trial reports. Its complete DNA sequence has been determined and published. It has excellent gastric acid resistance, bile tolerance, gut adherence and colonizing properties in newborn infants, children and adults.[11] Immunologically, it seems to promote Th1 response as shown clinically following vaccination against influenza[12] or in reducing the risk of respiratory tract infection in early childhood.[13] As known, immune response are polarized either versus a Th1-type or versus a Th2-type, being this last the typical pro-atopy response.[14] The BB12 strain has indeed anti-Th2 properties as shown in a report demonstrating the strain modified allergic inflammation.[15] BB12 can change the immune response so that it does not cause atopic manifestations, as clearly demonstrated in a neonatal gnotobiotic piglet disease model.[16] *E. faecium* L3 (L3) was originally isolated in the feces of a healthy newborn by Russian researchers who identified it through its ability to kill *Streptococcus agalactiae* by mean of enterocin (A and B) release.[17] Recently, a trial in Italy in pregnant women confirmed its ability to reduce swab test positivity to group B streptococcus while also decreasing the number of cases of premature rupture of membranes.[18] The L3 strain has been extensively used in newborn and preterm infants with positive effects on bodyweight, body mass index, respiratory infections, necrotizing enterocolitis and infective complications.[19-21] For these reasons we examined the ability of a mixture of BB12 and L3 to reduce the

signs and symptoms of atopy in children: *Bifidobacteria* are frequently depleted in atopic children[7] and adults,[8] and L3 promotes the preservation of endogenous gut *Bifidobacteria* in children.[10] The results of our retrospective clinical analysis show that this mixture of strains has beneficial effects in atopic children, especially when administered prophylactically at least 3 months before the appearance of the signs and symptoms of allergy and/or asthma. The fact that the mixture shows better results when administered prophylactically indicates that time is required for the probiotics to colonize the gut and exert their beneficial effects. We compared the results in the treated group with the results in the two control groups: the same children in the previous year (2016) and different children in the same year (2017). As atopy can improve or worsen on an annual basis due to variations in parameters such as weather conditions and pollen quantity, the use of two control groups reduced the risk of incorrect conclusions. As far as we are aware, there was less atopy in 2017 compared with 2016. However, in the prophylactic arm of the study, we did not find any differences in signs and symptoms of rhinitis, watery eyes and cough/bronchospasm between the treated group and the untreated group of the year before (2016) or between the treated and untreated groups of the same year (2017). It is difficult to interpret the findings in the treatment arm of the study because of the small number of children enrolled. Nevertheless, there was a clear tendency for the use of the probiotic mixture to reduce, even if only mildly significantly, the signs and symptoms of atopy. In order to obtain more data, we evaluated the odd ratios of having signs and symptoms of atopy and of using fewer conventional anti-atopy drugs by combining all participants from the prophylactic and treatment arms of the study. Better results regarding signs and symptoms were obtained by comparing different children in the same year (treated versus untreated in 2017), while better results regarding reduced drug use were obtained by comparing the same children in two different years (treated 2017 versus untreated 2016). We do not have a clear explanation for these differences but they may be due to an insufficient number of enrolled children, which is a possible source of bias. Another important limitation is the lack of blinding. A prospective rather than a retrospective approach would also have allowed a better explanation of the obtained results.

Although various publications have suggested that gut dysbiosis, often focusing on bifidobacterial species, may underlie atopy and several trials have been conducted where probiotics were administered to atopic subjects, there are few consistent results and meta-analysis does not clearly support to the benefits of probiotic use.[22-24]

Probiotics seem to be helpful in improving symptoms and quality of life in patients with allergy and/or asthma, but current evidence is strongly limited by study heterogeneity and the use of different outcome measures. Results are often contradictory and there are no guidelines on the use of probiotics to prevent or treat atopy. However, our study suggests that prophylactic administration of the correct probiotics could have a beneficial effect on atopy.

In conclusion, despite possible bias in our retrospective analysis, the prophylactic use in atopic children of a mixture of the widely documented probiotic strains BB12 and L3, reduced signs and symptoms of atopy such as rhinitis, watery eyes and cough/bronchospasm. This reduction resulted in decreased use of conventional drugs including oral anti-histamines, inhaled corticosteroids and adrenergic β2 agonists.

Conflict of interest

FDP is on the Scientific Board of Omeopiacenza, the firm supplying the product in Italy. None of the other Authors have a conflict of interest and none have received payment for writing this paper.

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Table 1
Characteristics of children in the prophylaxis arm of the study

Group	Sex	n	Age*	p
Untreated 2016	M	21	9.5 ± 2.8	N. S.
	F	14	10.1 ± 2.8	N. S.
Treated 2017	M	21	10 ± 3.3	N. S.
	F	14	11.1 ± 2.8	N. S.
Untreated 2107	M	18	8.1 ± 2.3	N. S.
	F	14	8.4 ± 3.4	N. S.

** Expressed in years (mean ± standard deviation); M: males; F: females; n: number of subjects; N. S.: not significant.*

Table 2

Signs and symptoms of atopy according to a visual analogue scale* in children in the prophylaxis arm of the study: comparison between treated and untreated children in the same year

Parameter	Untreated 2017	Treated 2017	Δ %	p
Rhinitis	2.3 ± 1.1	1.2 ± 1	-47.8	0.0001
Watery eyes	1.7 ± 1.1	0.8 ± 0.9	-52.9	0.0011
Cough/bronchospasm	2 ± 1.1	1.1 ± 1.1	-45.0	0.0019

** Expressed as mean ± standard deviation with: 0 = no signs; 1 = mild signs; 2 = moderate signs; 3 = severe signs.*

Table 3

Signs and symptoms of atopy according to a visual analogue scale* in children in the prophylaxis arm of the study: comparison between children treated in 2017 with the same subjects (untreated) in 2016

Parameter	2016	2017	Δ %	p
Rhinitis	2.5 ± 1.3	1.2 ± 1	-52.0	0.0001
Watery eyes	1.9 ± 1.5	0.8 ± 0.9	-57.9	0.0001
Cough/bronchospasm	1.9 ± 1.3	1.1 ± 1.1	-42.1	0.0002

**Expressed as mean ± standard deviation with: 0 = no signs; 1 = mild signs; 2 = moderate signs; 3 = severe signs.*

Table 4
Characteristics of children in the treatment arm of the study

Group	Sex	n	Age*	p
Untreated 2016	M	8	10.8 ± 3.5	N. S.
	F	3	8 ± 1	N. S.
Treated 2017	M	8	11.8 ± 3.5	N. S.
	F	3	9 ± 1	N. S.
Untreated 2107	M	7	9.9 ± 2.9	N. S.
	F	4	8.3 ± 3.8	N. S.

**Expressed in years (mean ± standard deviation); M: males; F: females; n: number of subjects; N. S.: not significant.*

Table 5

Signs and symptoms of atopy according to a visual analogue scale* in children in the treatment arm of the study: comparison between treated and untreated children in the same year

Parameter	Untreated 2017	Treated 2017	Δ %	p
Rhinitis	1.5 ± 0.9	0.4 ± 0.7	-73.3	0.0121
Watery eyes	0.9 ± 1.1	0.1 ± 0.3	-88.9	0.0358
Cough/bronchospasm	1.5 ± 1.4	0.6 ± 0.9	-60.0	0.11

** Expressed as mean ± standard deviation with: 0 = no signs; 1 = mild signs; 2 = moderate signs; 3 = severe signs.*

Table 6

Signs and symptoms of atopy according to a visual analogue scale* in children in the treatment arm of the study: comparison between children treated in 2017 and same subjects (untreated) in 2016

Parameter	2016	2017	Δ %	p
Rhinitis	1.1 ± 1.3	0.4 ± 0.7	-63.6	0.125
Watery eyes	0.8 ± 1.3	0.1 ± 0.3	-87.5	0.125
Cough/bronchospasm	2.1 ± 1.3	0.6 ± 0.9	-70.0	0.0156

** Expressed as mean ± standard deviation with: 0 = no signs; 1 = mild signs; 2 = moderate signs; 3 = severe signs.*

Table 7

Odd ratio (OR) in all children in the study: comparison between different subjects in the same year (untreated 2017 versus treated 2017)

Parameter	Untreated 2017	Treated 2017	p	OR (95% C. I.)
Rhinitis	0.9 ± 0.3	0.6 ± 0.5	0.004	0.2 (0.1-0.6)
Watery eyes	0.7 ± 0.4	0.4 ± 0.5	0.002	0.2 (0.1-0.6)
Cough/bronchospasm	0.8 ± 0.4	0.5 ± 0.5	0.005	0.2 (0.1-0.7)
Anti-histamine (oral)	0.7 ± 0.5	0.5 ± 0.5	0.09	0.5 (0.2-1.1)
Corticosteroid (oral)	0.1 ± 0.4	0 ± 0.1	0.044	0.5 (0.2-0.8)
Anti-leukotrienes (oral)	0.1 ± 0.3	0 ± 0.1	0.29	0.3 (0.1-3.0)
Corticosteroid (inhaled)	0.3 ± 0.5	0.3 ± 0.5	0.82	1.1 (0.5-2.7)
Adrenergic β2 agonists	0.3 ± 0.5	0.2 ± 0.4	0.26	0.6 (0.2-1.5)
Anti-histamine (eye drops)	0.1 ± 0.3	0.1 ± 0.3	0.85	1.1 (0.3-4.0)

**Expressed as mean ± standard deviation.*

Table 8

Odds ratio (OR) in all children in the study: comparison between the same subjects in different years (untreated 2016 versus treated 2017)

Parameter	Untreated 2016*	Treated 2017*	p	OR (95% C. I.)
Rhinitis	0.8 ± 0.4	0.6 ± 0.5	0.12	0.5 (0.2-1.2)
Watery eyes	0.6 ± 0.5	0.4 ± 0.5	0.07	0.5 (0.2-1.0)
Cough/bronchospasm	0.7 ± 0.4	0.5 ± 0.5	0.035	0.4 (0.2-0.9)
Anti-histamine (oral)	0.8 ± 0.4	0.5 ± 0.5	0.002	0.2 (0.1-0.6)
Corticosteroid (oral)	0 ± 0.2	0 ± 0.1	0.57	0.5 (0.1-5.6)
Anti-leukotrienes (oral)	0.1 ± 0.3	0 ± 0.1	0.18	0.2 (0.1-2.2)
Corticosteroid (inhaled)	0.6 ± 0.5	0.3 ± 0.5	0.008	0.3 (0.1-0.7)
Adrenergic β2 agonists	0.4 ± 0.5	0.2 ± 0.4	0.06	0.4 (0.2-0.6)
Anti-histamine (eye drops)	0.2 ± 0.4	0.1 ± 0.3	0.41	0.6 (0.2-1.9)

**Expressed as mean ± standard deviation.*

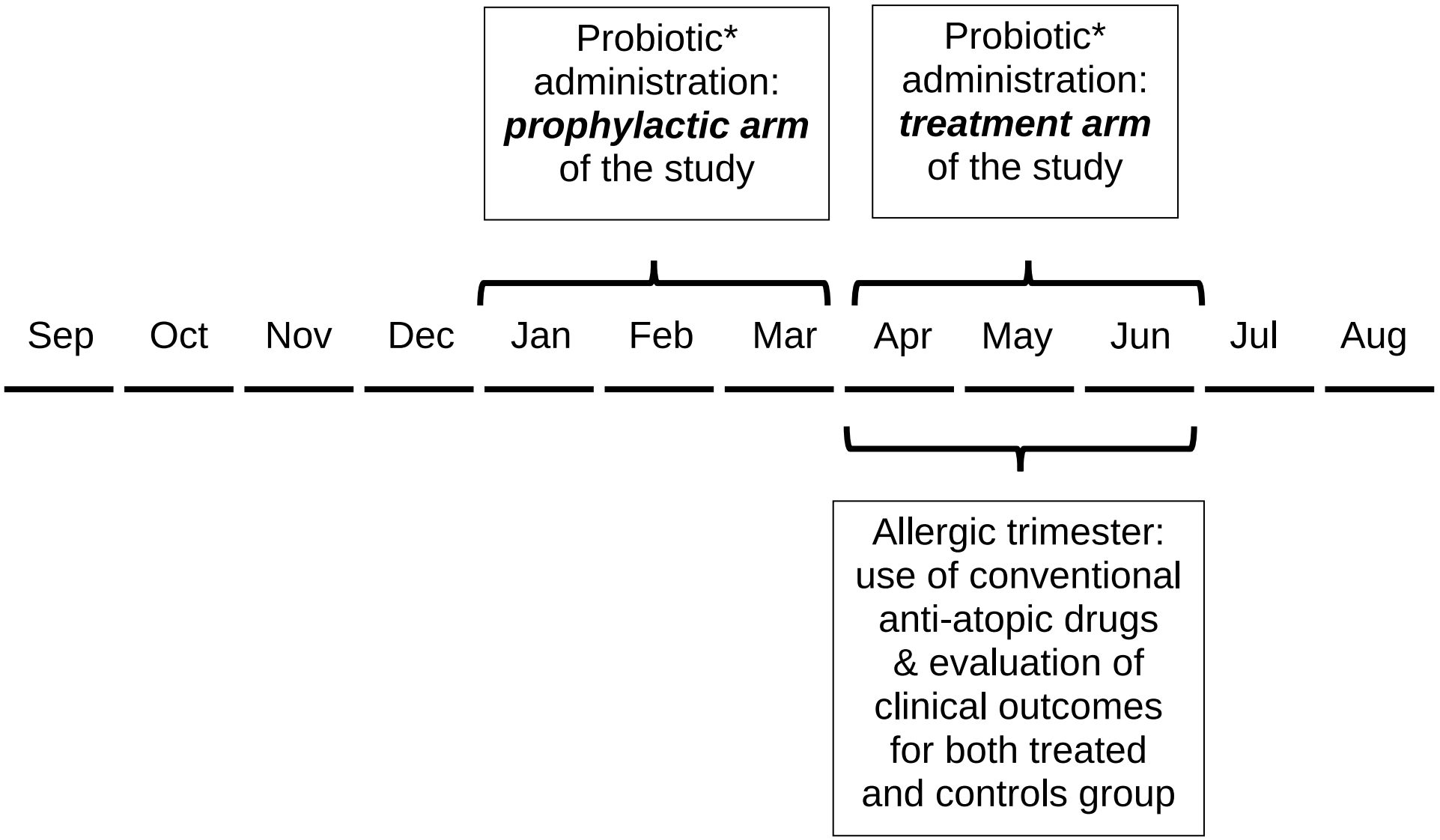

Figure 1 Study scheme
(* *Bifidobacterium animalis subsp. lactis* BB12 and *Enterococcus faecium* L3)