Pediatric RESEARCH

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CLINICAL RESEARCH ARTICLE

Effect of probiotics on thymus size and markers of infection in late infancy: a randomized controlled trial

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BACKGROUND: Probiotics are known to stimulate the immune system but the effect on thymus size in late infancy is unknown. We examined the effect of probiotics on thymus size and C-reactive protein (CRP) in healthy Danish infants starting daycare. We further examined associations between thymus size, CRP and recent infections.
METHODS: The study included 186 children randomized to a combination of *Lactobacillus rhamnosus*, LGG® and *Bifidobacterium animalis* spp. *lactis*, BB-12® or placebo for 6 months. Thymus size, assessed as thymus index (TI) and thymus weight index (TWI), was measured by ultrasound at baseline and at endpoint. Blood samples were drawn to measure CRP. Infections were parent-reported.
RESULTS: There was no significant difference in thymus size between the probiotic group and placebo ($p \geq 0.248$) but TWI tended to be higher in the probiotic group corresponding to 5% higher than placebo ($p = 0.068$) in an adjusted model. There was no effect of probiotics on CRP ($p = 0.331$). At the endpoint, thymus size was inversely associated with CRP ($p \leq 0.040$), diarrhea ($p \leq 0.050$), and TI was also associated with the absence from daycare due to respiratory or gastrointestinal infections ($p = 0.010$).
CONCLUSION: The probiotic intervention had no effect on thymus size or CRP in Danish children at the age of starting daycare.

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IMPACT:

- Overall there was no effect on thymus size of a combination of *Lactobacillus rhamnosus*, LGG® and *Bifidobacterium animalis* spp. *lactis*, BB-12® administered to Danish children starting daycare.
- This study examines the effect of probiotics on thymus size in healthy children when they start daycare thus exposed for infections while their immune system is still developing. This has to our knowledge not been described before.
- We found no significant difference in thymus size between the probiotic and placebo groups, but for thymus weight index, there was a trend. This should be investigated further in studies designed for this as primary outcome.

INTRODUCTION

The thymus is a primary lymphoid organ with many specialized functions of the immune system, including maturation and differentiation of T cells.[1] It starts to develop already in week 8 in pregnancy, and postnatally reaches its peak size relatively to the total body weight a short time after birth. At the age of 6 −8 months, the thymus has been reported to cease to grow and may temporally decrease in late infancy followed by growth until puberty where it starts to involute.[1–4]

Apart from age, the size of the thymus is also affected by nutrition. Breastmilk contains many components that are believed to enhance the immune system of the infant.[5,6] Several studies have shown that breastfed infants have larger thymus than those not breastfed. Infants exclusively breastfed at the age of 4 months and also infants partially breastfed in later infancy have larger thymus compared to formula-fed and infants no longer breastfed, respectively.[7,8] However, some studies have not found such an association.[1,9] Malnutrition in children has for a long time been known to cause thymus atrophy which is, however, reversible upon appropriate nutrition.[10,11] Moreover, the thymus size at birth and in infancy has been negatively associated with child mortality,[12,13] indicating that the size is important for the immune function and for development of a normally functioning immune system.[14] A small thymus has also been related to recent infection.[3,8] Thus, thymus size has been suggested as a proxy for its function.[12]

Probiotics may mediate development and stimulation of the immune system. In the recent years the effects of probiotics have been much studied regarding respiratory infections, diarrhea and other immune-related diseases.[15–19] However, the effect of probiotics on the thymus size during the first years of life, when the thymus reaches its maximal size and the immune system is still developing, is less explored. In a study by Indrio et al.,[20] infants who received fermented formula with probiotics had a larger thymus at 4 months after birth, compared to infants receiving standard formula. The impact of probiotics in later infancy on thymus size has, to our knowledge, not been investigated.

The aim of this study was to examine the effect of daily probiotic supplementation for 6 months in late infancy on thymus size in a randomized trial and further to investigate possible correlations between thymus size, recent infections and breast-feeding in late infancy. In addition, the effects of probiotics on

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C-reactive protein (CRP) as well as the association between thymus size and CRP were investigated.

METHODS

Study design and participants

The study was part of the ProbiComp intervention study; a randomized double-blinded placebo-controlled parallel trial with the primary outcome to investigate if probiotics, supplemented for 6 months to infants during the first months in daycare, could reduce absence from daycare due to infections.[21] The study was approved by the Committees on Biomedical Research Ethics for the Capital Region of Denmark (H-4-2014-032). Written informed consent was obtained from all parents and legal guardians of the children. The study was registered at clinicaltrials.gov (identifier NCT02180581, posted 02/07/2014).

The study has been described in detail elsewhere.[21] Briefly, participants were recruited from the capital region of Denmark during autumn seasons in 2014 and 2015. Inclusion criteria were term single-born infants with birthweight >2500 g, and expected to start daycare at the age of 8–14 months between September and February. Onset of the intervention was up to 12 weeks prior to expected start in daycare. Exclusion criteria were severe chronic illness, regular medication, antibiotic treatment within 4 weeks prior to baseline examination, and non-Danish-speaking parents. Intake of probiotic supplements or fermented milk products with probiotics was not allowed 2 weeks prior to the baseline examination and during the whole intervention period. Intake of other yoghurt products was limited to 1–2 meals per week. A total of 290 infants were included and randomized in blocks of 8 to receive either probiotics, consisting of a combination of *Lactobacillus rhamnosus*, LGG®, and *Bifidobacterium animalis* spp. *lactis*, BB-12® each in a dose of 10^9 colony-forming units per day, or maltodextrin as placebo. Both products were provided for free by Chr. Hansen A/S, Hørsholm, Denmark (LGG® and BB-12® are registered trademarks of Chr. Hansen).

Data collection

Participants were examined at baseline and at the end of intervention comprising anthropometry, sonographic measurement of thymus and a venous blood sample as well as structured interviews of the parents including questions regarding breastfeeding. Full breastfeeding allowed the child to have a maximum of one meal of formula per week or was otherwise breastfed. Occurrence of illness during the intervention was registered by the parents using daily and weekly web-based questionnaires. In this study, recent infections during the last month of intervention were covered and included the following variables: number of days absent from daycare due to respiratory or gastrointestinal infections, days with diarrhea, cold symptoms and fever during the last month of the intervention.

Thymus size measurement

Thymus size was measured by ultrasound as described in detail earlier[22,23] using a scanner (Sonoscape A6 Diagnostica, Otterup, Denmark) with a pediatric abdominal probe. Thymus size was estimated using sonographic measures of the transverse diameter of the thymus and the sagittal area of its largest lobe, and both measures were performed twice when possible. The mean of the two measures was multiplied to give a measure of the thymus index (TI). The thymus weight index (TWI) was calculated to adjust for body weight by dividing TI with infant body weight, thus representing absolute and relative thymus size, respectively.[24] The thymus was measured by three examiners. In the first season, examiners 1 and 2 performed the scans at baseline and examiners 1 and 3 at the endpoint examinations. In the second season, examiners 1 and 3 performed the scans at baseline and examiners 1 and 2 at endpoint. To adjust for interobserver variation, a variable combining thymus examiner at baseline and endpoint was generated and included in the statistical models.

Blood samples

A venous blood sample was drawn and collected in serum tubes. After preparation, blood samples were stored at −80 °C until analyses. High-sensitive C-reactive protein (CRP) was measured using ABX Pentra® 400 (Horiba ABX Dianogstics, Kyoto Japan (DK; Trilab)). Limit of detection was <0.12 mg/L and samples below this value (visit 1: $n = 44$; visit 2: $n = 36$) were coded as 0.055 mg/mL.

Statistics

Descriptive statistics stratified by the intervention group are given as mean ± SD and median (IQR) for parametric and nonparametric continuous variables, respectively, while categorical variables are given as *n* (%). Comparisons between sex, breastfeeding status and intervention groups at baseline only were tested by independent *t* test, Mann−Whitney *U* test or chi-square test as appropriate. Changes over time within groups were analyzed by paired *t* test.

The effect of probiotics on thymus size measured as TI and the weight-adjusted variable TWI was assessed using the linear mixed model. The crude model was adjusted for sex and baseline value, and a variable combining thymus examiner at baseline and endpoint was generated and applied as a random factor in the model. The fully adjusted model included further adjustment for age and breastfeeding status at baseline and at endpoint, intervention year, number of days of intervention and compliance. The effect of probiotics on CRP was analyzed by general linear models using log-transformed CRP values and adjustment for sex and baseline value in the crude model. The fully adjusted model was further controlled for breastfeeding, age, intervention year, duration of intervention and compliance as for thymus size. Model assumptions were checked using residual plots.

Correlations between thymus size and outcomes were investigated using all children by pooling the groups. Associations between thymus size and recent infections were investigated using the linear mixed models adjusted for age, sex, baseline and thymus examiner. Spearman's rho was used to explore the correlations between thymus size or change in thymus size and duration of full breastfeeding or CRP and between CRP and recent infections. Significance was defined as p values < 0.05 and trends as p values < 0.10. Data were analyzed using IBM SPSS Statistics (version 22, IBM, New York. USA).

RESULTS

Of the 290 infants included in the study, 260 completed the study. Of these, 186 infants had measurements of thymus size at both baseline and endpoint (97 and 89 in the probiotic and placebo group, respectively). There was no difference in baseline characteristics between infants that completed the study with or without thymus measurement. At baseline, the probiotic group had a larger TI and TWI but lower weight-for-length-SDS (WLZ). Otherwise, there were no differences between the groups (Table 1).

During the 6-month intervention, the absolute and relative thymus size were diminished corresponding to about 15% for TI and 25% for TWI ($p \le 0.001$) for both groups (Fig. 1). TI was decreased to 15.71 ± 4.07 and 14.54 ± 3.15 cm^3 and TWI to 1.46 ± 0.33 and 1.32 ± 0.28 cm^3/kg for probiotics and placebo, respectively.

At the end of intervention, the TI did not differ between the groups ($p = 0.248$), whereas there was a trend for a larger TWI in the probiotic group compared to placebo corresponding to a 5% higher TWI ($p = 0.083$) in the crude model adjusted for sex, baseline value and thymus examiner. The estimated means (CI) were 1.42 (1.35–1.50) cm^3/kg vs. 1.35 (1.27–1.43) cm^3/kg for the

probiotic and the placebo group, respectively. In the fully adjusted models including i.e. breastfeeding, age and compliance, the trend was accentuated for TWI whereas there was no trend or effect for TI ($p = 0.068$ and $p = 0.220$, respectively).

Of the infants with thymus data, CRP measurement was available for 142 and 150 infants at baseline and at the end of study, respectively, and 114 infants had measurements at both examinations. Intake of probiotics did not alter the CRP value compared to placebo ($p = 0.331$) in crude models adjusted for sex and baseline value or in the fully adjusted model ($p = 0.485$). There was still no difference between groups if only infants with CRP values above the detection limit (i.e. indication of a recent infection) at both start and end of intervention were included ($n = 58$, $p = 0.665$). This was also found in the fully adjusted model ($p = 0.525$).

Table 1. Characteristics of the participants according to the intervention group (values are mean ± SD, median (IQR) or *n* (%)).

	Probiotics $n = 97$	Placebo $n = 89$	p value[a]
Baseline			
Girls (*n* (%))	42 (43)	44 (49)	0.402
Age (months)	10.0 ± 0.8	10.0 ± 0.8	0.892
Weight (kg)	9.38 ± 1.08	9.55 ± 0.93	0.249
Length (cm)	73.7 ± 2.6	73.7 ± 2.6	0.849
Weight-for height-SDS	0.30 ± 0.91	0.57 ± 0.79	0.029
Weight-SDS	0.44 ± 0.89	0.66 ± 0.80	0.087
Length-SDS	0.54 ± 0.93	0.56 ± 1.02	0.858
Currently breastfed (*n* (%))	50 (51.5)	39 (43.8))	0.292
Thymus/CRP outcomes			
Thymus index (cm^3)	18.27 ± 3.93	17.13 ± 3.50	0.040
Thymus weight index (cm^3/kg)	1.96 ± 0.40	1.80 ± 0.36	0.006
CRP (mg/L)[b]	0.13 (0.06; 0.42)	0.14 (0.06;0.68)	0.495
Study characteristics			
Compliance (%)	95 ± 5	95 ± 6	0.869
Duration of intervention (days)	186 ± 5	186 ± 5	0.344
Duration of full breastfeeding (months)	5.00 (2.60; 5.46)	4.00 (2.35; 5.08)	0.073
Breastfed at end of intervention (*n* (%))	11 (11.3)	6 (6.7)	0.277

[a]Difference between probiotic group and placebo group by *t* test, Mann−Whitney or chi-squared.
[b]Probiotics, $n = 66$; Placebo, $n = 76$.

We also explored the relation of potential influencing factors, i.e. breastfeeding, sex, recent infections and CRP levels, with thymus size. Overall, only 17 infants (8 girls) were still breastfed at the end of the intervention and there were no differences between infants still breastfed and no longer breastfed for TI ($p = 0.779$) or TWI ($p = 0.711$). The groups were pooled and correlations between thymus size and duration of full breastfeeding were investigated in all children. There was a trend for a positive correlation between duration of full breastfeeding and change in TI or TWI over the 6 months of intervention, i.e. the decrease in TI or TWI tended to be less in infants fully breastfed for a longer period ($r = 0.139$, $p = 0.059$ and $r = 0.135$, $p = 0.066$, for TI and TWI, respectively).

Boys had higher TI at both examinations (mean (SD) 18.66 (3.89) cm^3 vs. 16.63 (3.30) cm^3 ($p \le 0.001$) and 15.70 cm^3 vs. 14.51 cm^3 ($p = 0.025$)) for boys versus girls at baseline and endpoint, respectively. Boys had higher TWI at baseline (1.94 (0.40) cm^3 vs. 1.82 (0.36) ($p = 0.042$)), but at the end of intervention, the sex difference had vanished for TWI (1.41 (0.33) cm^3 vs. 1.37 (0.29) cm^3 ($p = 0.40$)).

The associations between infections during the last month and thymus size at the end of intervention were assessed using adjusted models (Table 2). The number of children who were absent from daycare due to respiratory or gastrointestinal infections in this period was 108 and the median (IQR) days of absence for these children was 3.0 (2.0; 4.0) days. Further, 23 children had diarrhea (median (IQR) 2.0 (1.0; 2.0) days), whereas 132 and 114 children had cold symptoms or fever, respectively (median (IQR) 5.0 (3.0; 8.0) and 3.0 (2.0; 4.0) days, respectively). TI was negatively associated with days absent from daycare due to infections and with days with diarrhea corresponding to 0.236 cm^3 smaller TI per day absent from daycare or 0.561 cm^3 per day with diarrhea. The associations were attenuated when the weight-corrected index TWI was used showing borderline significance. There were no associations between thymus size and infections during the 6-month intervention period.

It was also investigated if CRP correlated with thymus size or change in thymus size during the intervention in the pooled

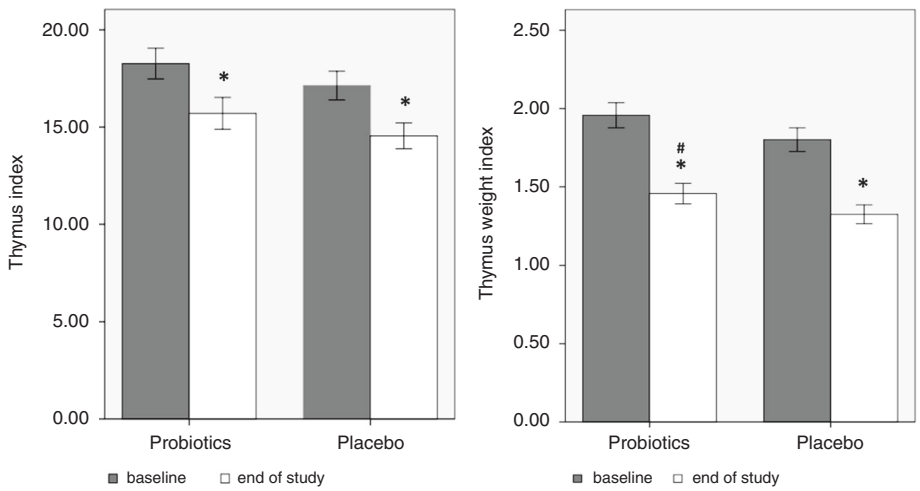

Fig. 1 Mean thymus index (cm^3) (left panel) and mean thymus weight index (cm^3/kg) (right panel) at baseline and end of study after 6-month intervention. Error bars represent 95% CI. *Different from baseline after 6-month intervention by paired *t* test ($p \le 0.001$). #Trend for difference between intervention groups by mixed linear model analyses adjusted for sex, baseline value and thymus examiner ($p = 0.083$).

Table 2. Associations between thymus size and days absent from daycare or days with infections during the last month. (Linear mixed models adjusted for sex, age, thymus size at baseline and thymus examiner. Values are beta estimate and 95% confidence interval, $n = 186$).

	Thymus index (cm^3)		Thymus weight index (cm^3/kg)	
	B (95% CI)	*p* value	*B* (95% CI)	*p* value
Absence due to respiratory or gastrointestinal infections (days)	−0.236 (−0.414 to −0.058)	0.010	−0.015 (−0.031 to 0.001)	0.062
Diarrhea (days)	−0.561 (−1.106 to −0.015)	0.044	−0.048 (−0096 to 0.000)	0.050
Cold (days)	−0.042 (−0.109 to 0.025)	0.219	−0.004 (−0.009 to 0.002)	0.238
Fever (days)	−0.155 (−0.345 to 0.034)	0.108	−0.008 (−0.025 to 0.009)	0.345

sample. At baseline there was no correlation between CRP and any of the thymus measures. At the end of intervention there was a negative association between CRP and TI (Spearman's rho = −0.174, $p = 0.033$, $n = 150$) which remained significant when correcting for infant weight (TWI, Spearman's rho = −0.168, $p = 0.040$). There was also a negative correlation between CRP at endpoint and change in thymus size during the intervention (Spearman's rho = −0.202, $p = 0.013$ and Spearman's rho = −0.194, $p = 0.017$, for TI and TWI, respectively) i.e., the higher the CRP value, the more pronounced the decrease in thymus size during the intervention. Furthermore, we found a positive correlation between CRP and sick days due to infections during the last month (Spearman's rho = 0.178, $p = 0.030$).

DISCUSSION

We have investigated the effect of a daily intake of a combination of the two probiotic strains BB-12® and LGG® or placebo for 6 months on thymus size in infants aged 8−14 months before starting daycare, as a secondary outcome in the ProbiComp Study.[21] Overall, there was no effect of the probiotic supplementation on thymus size but there was a trend for a larger TWI corresponding to 5% when compared to the placebo group. This may indicate that the effect depends on body size, and the relative thymus size is also important to consider as the body size might blur the effect. There was no difference between groups in days absent from daycare due to respiratory or gastrointestinal infections which was the primary outcome in the main study[21] but there was a reduced incidence of eczema in the probiotic group.[25]

The effect of probiotics on thymus size in infants during the first years of life when the thymus is under development is not widely examined. Indrio et al. investigated the effect of a fermented formula containing *Bifidobacterium breve C50* and *Streptococcus thermophilus 065* on thymus size and compared it to infants receiving standard formula and a reference group of breastfed infants in infants up to 4 months after birth.[20] They found that intake of the fermented formula with probiotics resulted in larger thymus size measured as TI compared to standard formula but not larger than those breastfed, thus supporting the hypothesis that probiotics might have a positive effect on thymus size. However, it is difficult to compare the study with the present study as they are not quite comparable. Besides different probiotic strains and doses, the infants in the present study were older, the diet was much more varied, and the infants were often breastfed in both intervention groups especially at baseline. On the contrary, in the study by Indrio et al., no infants were breastfed which may influence the results as breastfeeding may have an impact on thymus size.[7,8] Further, the infants were about to start daycare at baseline in our study, and thus during the intervention they were exposed to more bacteria and vira which also may have an influence on thymus size.[9,26] However, it is interesting if the trend for TWI observed in our study indicates a real positive effect on thymus size. This would be in accordance with the positive effect described by Indrio et al., suggesting a broader age range where ingestion of probiotic may have a positive effect on thymus size.

Another study investigating the same strain of probiotics as ours did not find any effect on thymus recovery measured as its increase in size in children aged 6–59 months with severe acute malnutrition in Uganda.[26] However, the study differed substantially from the present study especially regarding the health, nutritional status and living conditions of the study population.

As mentioned previously, the literature on probiotics and thymus size is very scarce and a potential change in thymus size due to probiotics may mainly be measurable in early life. However, thymus size has been shown to correlate with T-lymphocyte subsets (e.g. CD4+ and CD8+) in infancy,[27] thus reflecting the thymus function and linking thymus size to thymus output. Also, thymus size has been reported to correlate with T-cell receptor rearrangement circles (TRECs), which is increasingly used as a measure of thymus output.[28,29] T-lymphocyte subsets have been used in several studies investigating the effect of probiotics on different aspects of the immune function, for example, in studies investigating the effect of probiotics on atopic dermatitis,[30] cow's milk allergy,[31] and function of lymphocytes in late preterm infants.[32] Various mechanisms for the modulation of the immune system by probiotics have been suggested, including gut immune barrier function.[33] Also an animal study showed that probiotic fermented milk enhanced the level of some cytokines that stimulate thymus epithelial cell proliferation and activity of thymocytes.[34] However, the potential effect on thymus size is not clear and needs further research.

Supplementation with probiotics had no effect on CRP levels compared to the placebo group. We would have expected that the probiotics would have a beneficial effect on the immune system showing lower CRP values compared to placebo. For many of the infants the CRP level was below the detection level; i.e. only 61% had measurable CRP values at both examinations. This low number of children with measurable CRP values and the general low CRP values indicate that although the infants are more exposed to risk for infections at the time of starting daycare and simultaneously stopping being breastfed, these Danish infants are overall well-nourished and healthy. To our knowledge, no other studies have explored the effect of probiotics on CRP during the first years of living.

We combined the two groups to investigate possible correlates of thymus size. The change in TI and TWI during the 6 intervention months in late infancy was inversely associated with CRP levels at the endpoint. This was expected as it has previously been reported that the size of the thymus is diminished by infections.[9,26] This is also in accordance with the inverse associations found between thymus size and recent infections the last month as respiratory or gastrointestinal infections and days with diarrhea. We found no associations with symptoms of cold or fever, which could be due to the fact that these are relatively mild infections not having a larger impact on thymus size. However, Hasselbalch et al.[3] found smaller TI at 12 months in Danish infants with fever episodes between 10 and 12 months but it was dependent on age as no comparable relation was observed for 8 and 10 months. Moreover, it was a small study; only 37 infants were included, and much larger studies might be needed.

Nevertheless, the negative associations between thymus size and infections and CRP values are in accordance with previous findings in the literature.[4,9,24,26] In the study by Birk et al.,[24] the thymus size at birth predicted episodes of infection up to the age of 3 months, whereas it is often reported that infections result in a smaller thymus size.[3,35] Our results thus support the negative relation between thymus size and infections but as it is based on associations the causality is not known. The exact mechanisms involved are not known and it may vary with the kind of infection. Thus, both local and systemic effects may be involved. The systemic effects may be mediated by glucocorticoids and proinflammatory factors, e.g., HIV infection causes a decrease in proliferating thrombocytes, but is reversible by prescribing antiretroviral therapy.[36–39]

We also investigated the association between breastfeeding and thymus size at the end of intervention, i.e. where the mean age of the children was 16 months and very few were still breastfed. It is well described in the literature that thymus size is larger in breastfed infants compared to formula-fed in early infancy.[7,8] Also in late infancy at 10 months where the breastfeeding rate is less intensive, there might be a difference as shown in a study by Hasselbalch et al.,[8] though Yekeler et al.[1] found nonsignificant higher TI of breastfed infants in infants up to 6 months. We have previously reported a larger TWI in girls still breastfed compared to girls who were no longer breastfed at baseline at the age of 10 months.[23] At the end of intervention very few infants were still breastfed and no difference in thymus size according to breastfeeding status was found. This might be due to the small sample size as only 17 children were still breastfed or the effect might diminish with time as breastfeeding was more intense at baseline. This may also be the reason why we only see a trend for an inverse association between change in thymus size and the duration of exclusive breastfeeding after pooling the breastfeeding groups. The positive correlation between duration of breastfeeding and change in TI or TWI during the intervention period is in accordance with a previous study showing that after a decrease in TI between 8 and 12 months, a group of exclusively breastfed infants increased very slowly in TI up to the age of 24 months whereas formula-fed and partially breastfed infants remained stable at this lower TI up to 24 months, i.e. the final decrease in TI was diminished by exclusive breastfeeding.[40]

The general decrease in thymus size during the 6-month intervention of the children with a mean age of 10 months at baseline was expected, and was thus in accordance with previous findings describing a decrease in thymus size at this age range.[1,3,4]

The strengths and limitations of the ProbiComp intervention study have been described elsewhere.[21] A limitation of the present study is the reduced sample size as not all infants had thymus measurements at both start and end of intervention. Nevertheless, as there was no difference in baseline characteristics between children with two thymus measurements and children with one or no thymus measurements, it is unlikely that this has affected the results. We found a trend for a higher TWI in the probiotic group which could be a chance finding. With a higher sample size, it might have been possible to detect a significant difference, but this is speculative. However, the ProbiComp study showed a reduction in the incidence of eczema in the probiotic group, indicating that there might be an influence on the immune system[25] but whether there is a link to thymus size remains to be investigated further. Markers of thymus function such as TRECs, CD4+ and CD8+ would provide further information on how the thymus might be affected and should be included in future studies.

To summarize, this is to our knowledge the first randomized trial examining the effect of probiotics on thymus size in late infancy in a high-income country. There was no significant effect of administration of probiotics on thymus size but we found a trend for a higher TWI. To further investigate the impact of probiotics on thymus size in healthy infants, future studies designed for this are warranted.

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AUTHOR CONTRIBUTIONS

Conception and design of the study: R.P.L., A.L., C.M., and K.F.M. Acquisition of data: R.P.L. Statistical analysis: A.L. Analysis and interpretation of data: A.L., K.F.M., M.J.H.R., C.M., and R.P.L. Drafting of the manuscript: A.L. All authors critically revised the manuscript and approved the final version of the manuscript.

ADDITIONAL INFORMATION

Competing interests: K.F.M. and C.M. received a grant from Chr. Hansen for the current study and for another study with probiotics in Ugandan children. The other authors have no financial relationships or potential conflicts of interest relevant to this article to disclose.

Consent statement: Written informed consent was required and obtained from all parents and legal guardians of the participating children.

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REFERENCES

1. Yekeler, E. et al. Analysis of the thymus in 151 healthy infants from 0 to 2 years of age. *J. Ultrasound Med.* **23**, 1321–1326 (2004).
2. Moore, S. E. et al. Thymic size is increased by infancy, but not pregnancy, nutritional supplementation in rural Gambian children: a randomized clinical trial. *BMC Med.* **17**, 38 (2019).
3. Hasselbalch, H., Jeppesen, D. L., Ersbøll, A. K., Engelmann, M. D. & Nielsen, M. B. Thymus size evaluated by sonography. A longitudinal study on infants during the first year of life. *Acta Radiol.* **38**, 222–227 (1997).
4. Moore, S. E. et al. Thymus development and infant and child mortality in rural Bangladesh. *Int. J. Epidemiol.* **43**, 216–223 (2014).
5. Field, C. J. The immunological components of human milk and their effect on immune development in infants. *J. Nutr.* **135**, 1–4 (2005).
6. Hossny, E. M. et al. Breast milk interleukin-7 and thymic gland development in infancy. *Eur. J. Nutr.* **59**, 111–118 (2020).
7. Hasselbalch, H., Jeppesen, D. L., Engelmann, M. D., Michaelsen, K. F. & Nielsen, M. B. Decreased thymus size in formula-fed infants compared with breastfed infants. *Acta Paediatr.* **85**, 1029–1032 (1996).
8. Hasselbalch, H., Engelmann, M. D., Ersboll, A. K., Jeppesen, D. L. & Fleischer-Michaelsen, K. Breast-feeding influences thymic size in late infancy. *Eur. J. Pediatr.* **158**, 964–967 (1999).
9. Rytter, M. J. H. et al. Correlates of thymus size and changes during treatment of children with severe acute malnutrition: a cohort study. *BMC Pediatr.* **17**, 70 (2017).
10. Chevalier, P. et al. Study of thymus and thymocytes in Bolivian preschool children during recovery from severe protein energy malnutrition. *J. Nutr. Immunol.* **3**, 27–39 (1994).
11. Prentice, A. M. The thymus: a barometer of malnutrition. *Br. J. Nutr.* **81**, 345–347 (1999).
12. Aaby, P. et al. Thymus size at birth is associated with infant mortality: a community study from Guinea-Bissau. *Acta Paediatr.* **91**, 698–703 (2002).
13. Garly, M.-L. et al. Thymus size at 6 months of age and subsequent child mortality. *J. Pediatr.* **153**, 683–688.e3 (2008).
14. Domínguez-Gerpe, L. & Rey-Méndez, M. Evolution of the thymus size in response to physiological and random events throughout life. *Microsc. Res. Tech.* **62**, 464–476 (2003).
15. Binns, C. & Lee, M. K. The use of probiotics to prevent diarrhoea in young children attending child care centres. *J. Exp. Clin. Med.* **2**, 269–273 (2010).
16. King, S., Glanville, J., Sanders, M. E., Fitzgerald, A. & Varley, D. Effectiveness of probiotics on the duration of illness in healthy children and adults who develop

common acute respiratory infectious conditions: a systematic review and meta-analysis. *Br. J. Nutr.* **112**, 41–54 (2014).
17. Pelucchi, C. et al. Probiotics supplementation during pregnancy or infancy for the prevention of atopic dermatitis: a meta-analysis. *Epidemiology* **23**, 402–414 (2012).
18. Szajewska, H., Skórka, A., Ruszczyński, M. & Gieruszczak-Białek, D. Meta-analysis: Lactobacillus GG for treating acute gastroenteritis in children-updated analysis of randomised controlled trials. *Aliment. Pharmacol. Ther.* **38**, 467–476 (2013).
19. Wang, Y. et al. Probiotics for prevention and treatment of respiratory tract infections in children. *Medicine* **95**, e4509 (2016).
20. Indrio, F., Ladisa, G., Mautone, A. & Montagna, O. Effect of a fermented formula on thymus size and stool pH in healthy term infants. *Pediatr. Res.* **62**, 98–100 (2007).
21. Laursen, R. P. et al. Probiotics and child care absence due to infections: a randomized controlled trial. *Pediatrics* **140**, e20170735 (2017).
22. Hasselbalch, H., Jeppesen, D. L., Ersbøll, A. K., Lisse, I. M. & Nielsen, M. B. Sonographic measurement of thymic size in healthy neonates. Relation to clinical variables. *Acta Radiol.* **38**, 95–98 (1997).
23. Laursen, R. P. et al. Thymus size is associated with breastfeeding and having pets in a sex-specific manner. *Acta Paediatr*. **109**, 968–975 (2020).
24. Birk, N. M. et al. Bacillus Calmette-Guérin vaccination, thymic size, and thymic output in healthy newborns. *Pediatr. Res.* **81**, 873–880 (2017).
25. Schmidt, R. M. et al. Probiotics in late infancy reduce the incidence of eczema: a randomized controlled trial. *Pediatr. Allergy Immunol.* **30**, 335–340 (2019).
26. Nabukeera-Barungi, N. et al. Thymus gland size during recovery from complicated severe acute malnutrition: a prospective study of the role of probiotics. *Paediatr. Int. Child Health* **39**, 95–103 (2019).
27. Jeppesen, D. L., Hasselbalch, H., Lisse, I. M., Ersbøll, A. K. & Engelmann, M. D. M. T-lymphocyte subsets, thymic size and breastfeeding in infancy. *Pediatr. Allergy Immunol.* **15**, 127–132 (2004).
28. Moore, S. et al. Early-life nutritional and environmental determinants of thymic size in infants born in rural Bangladesh. *Acta Paediatr.* **98**, 1168–1175 (2009).
29. Ngom, P. T. et al. Improved thymic function in exclusively breastfed infants is associated with higher interleukin 7 concentrations in their mothers' breast milk. *Am. J. Clin. Nutr.* **80**, 722–728 (2004).
30. Gerasimov, S. V., Vasjuta, V. V., Myhovych, O. O. & Bondarchuk, L. I. Probiotic supplement reduces atopic dermatitis in preschool children: a randomized, double-blind, placebo-controlled, clinical trial. *Am. J. Clin. Dermatol.* **11**, 351–361 (2010).
31. Hol, J. et al. The acquisition of tolerance toward cow's milk through probiotic supplementation: a randomized, controlled trial. *J. Allergy Clin. Immunol.* **121**, 1448–1454 (2008).
32. Zhang, S.-F. et al. Effects of clostridium butyricum and bifidobacterium on BTLA expression on CD4+ T cells and lymphocyte differentiation in late preterm infants. *Microb. Pathog.* **100**, 112–118 (2016).
33. Maldonado Galdeano, C., Cazorla, S. I., Lemme Dumit, J. M., Vélez, E. & Perdigón, G. Beneficial effects of probiotic consumption on the immune system. *Ann. Nutr. Metab.* **74**, 115–124 (2019).
34. Núñez, I. N. et al. Effect of a probiotic fermented milk on the thymus in Balb/c mice under non-severe protein-energy malnutrition. *Br. J. Nutr.* **110**, 500–508 (2013).
35. Jeppesen, D. L., Ersbøll, A. K., Nielsen, S. D., Hoppe, T. U. & Valerius, N. H. Low thymic size in preterm infants in the neonatal intensive care unit, a possible marker of infection? A prospective study from birth to 1 year of age. *Acta Paediatr.* **100**, 1319–1325 (2011).
36. Dion, M.-L. et al. HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation. *Immunity* **21**, 757–768 (2004).
37. Lee, J. C. et al. Thymic volume, T-cell populations, and parameters of thymopoiesis in adolescent andadult survivors of HIV infection acquired in infancy. *AIDS* **20**, 667–674 (2006).
38. Nunes-Alves, C., Nobrega, C., Behar, S. M. & Correia-Neves, M. Tolerance has its limits: how the thymus copes with infection. *Trends Immunol.* **34**, 502–510 (2013).
39. Savino, W. & Dardenne, M. Nutritional imbalances and infections affect the thymus: consequences on T-cell-mediated immune responses. *Proc. Nutr. Soc.* **69**, 636–643 (2010).
40. Hasselbalch, H., Ersbøll, A. K., Jeppesen, D. L. & Nielsen, M. B. Thymus size in infants from birth until 24 months of age evaluated by ultrasound. A longitudinal prediction model for the thymic index. *Acta Radiol. Stockh. Swed. 1987* **40**, 41–44 (1999).