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# A non-hydrolyzed, fermented milk formula reduces digestive and respiratory events in infants at high risk of allergy

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Dear Pr Prakash Shetty,

Please find enclosed a second revision of the manuscript reference 2009EJCN0426R, entitled “*A non-hydrolyzed, fermented milk formula reduces digestive and respiratory events in infants at high risk of allergy.*” This manuscript was revised according to the reviewers’ comments.

You will find in another document our point-by-point answers to reviewer comments.

We hope this will agree the referee and the revised manuscript will be accepted for publication.

Sincerely yours,

Christophe Dupont

## RESPONSE TO REVIEWER'S COMMENTS

Morisset et al., 2009EJCN0426R

### Reviewer #1 (Comments to the Author):

The authors of this paper have given a full response to my comments, taking on board many of my suggestions. There are still a few areas which need further clarification, or in some cases the clarification needs to be made in the text of the paper as well as in their response to my comments.

**Point 1: Selection of study participants - How were the 136 children selected for study? What was the sampling frame or other method used to identify the high risk families?**

As stated in the Inclusion criteria section, page 6, they were selected as follows: *“family at risk of atopy: at least two atopic members, including at least one of the parents (i.e. mother and/or father and/or at least one brother or sister).”*

**Point 2: The abstract describes this as a "multicentre, randomized, double-blind, controlled study" study, but this description is not apparent in the manuscript itself.**

Presence of two centres and 16 private investigators is now clearly stated in the Subjects section, just before description of the randomization method.

**Point 3: Response rate - My previous comment about the response rate refers to the calculation of the percent of randomized subjects who completed the study in each study group. The authors do have these numbers.**

Response rates were as follows: SF:  $55/63=87.3\%$  and HKBBST:  $53/66=80.3\%$ . These figures are now included in the first paragraph of the Results section.

**Point 4: Withdrawal from study - I notice that 3 children were withdrawn due to "unknown date of weaning". Was this necessary given that this variable is not used in any of the analysis (other than to describe the characteristics of the children)?**

This exclusion criterion was part of the protocol. Indeed, in the absence of weaning date, we could not be certain that the formulation had actually been used, and we could not calculate any duration of use. This is now indicated in the Inclusion criteria section.

**Point 5: Blinding - The authors have now stated that the allergist and dietician were blinded to the intervention product. Was the mother also blinded? Even though the study was "double-blinded" it is still necessary to make it clear that the mother, as well as the health professionals were blind to the study product (if indeed this was the case).**

In this double-blind, mothers were obviously blind to the intervention product. This is now stated in the Product-under-study section of the manuscript.

**Point 6: Compliance with study formulae - The authors have now stated that "the dietician checked diet compliance and tolerance by the mothers". Was child compliance also checked? Was use of non-study formula by the child recorded in the diary? It would be strengthen the manuscript if the authors could provide some data on compliance for both breast feeding mothers and the child. Non or partial compliance would weaken any effect of the HKBBST formula.**

Compliance of the mother and child was asked by the dietician to the mother during phone interviews at 4 and 12 months. No stop of the products was reported. However, use of other

prebiotic- or probiotic-containing dairy products, and use of any other milk or formula was reported in the diary and analyzed. All mothers, being in a family prone to allergy, were willing to stick to the formula, hoping that it was the "active" one. The dietician and the research nurse, who knew exactly which amount of formula was necessary, checked whether orders for additional formula were adequate or not, with a report to the CRO at each visit. The following data were observed, which confirm that use of other milk was low and similar between groups. This data is now included in the first paragraph of the Results section.

	Standard	HKBBST	Total
<b>No use of another milk at 4 months</b>	82%	81%	81.5%
<b>No use of another milk at 12 months</b>	67%	75%	71.3%

Groups were not significantly different for this criterion.

**Point 7: Exposure - The length of the intervention i.e. 1 year, needs to be explicitly stated in the manuscript and the abstract.**

We agree with this comment and have included this precision in abstract and methods.

**Point 8: Outcomes - I thank the authors for providing further explanation on the relationships between the different study outcomes (skin prick test, patch test, blood test, oral challenge). However I think this needs further clarification in the manuscript itself. Perhaps the best way to do this would be as a flow diagram showing the decision-making process for each test and the numbers undergoing each test.**

We agree that a diagram would better illustrate the decision-making process and have included it in the manuscript (Figure 1). Nonetheless, the number of figures being limited to 6, and since the current number of figures was already of 6 (including sub-parts of figure 2), including this figure requires authorization by the editor.

**Point 9: There is also a slight discrepancy between the authors' response to reviewers, which states that "IgEs were performed when an "allergic event" (ie PAAE) was suspected", whereas the manuscript states that "blood was sampled when an allergic event had occurred ...". I suspect the former is correct if based on a positive PAAE. Can the authors clarify please?**

The former is correct, as now indicated in the text.

**Point 10: Also what are "reintroduction tests"? It is unfortunate that in some sub-group analyses, e.g. IgE analyses, the numbers are very small. Some results would have been more conclusive if the sample size had been larger.**

Figure 1 now shows precise decision diagram. IgE quantification only occurred in case of suspected PAAE. This allowed reducing the number of blood samplings in these very young children, and explains the low number of children with IgE results.

Reintroduction tests were, actually, Home Food Challenges (Fig 1 and Text page 10), i.e. the procedure according to which milk was gradually reintroduced in the diet after OFC, in order to detect potential delayed allergy. Changes have been made.

The study was started when the lower level of detectability for specific IgE was 0.35KU/L. The detection threshold was decreased to 0.10 during the study and allowed detecting positivity in some children. If this modification had occurred before the study we might have considered systematic blood sampling.

**Point 11: The results, provided in the response to reviewers, for the differences between**

study groups in children having at least one PAAE, should be included in the manuscript. They are interesting because while there were differences between study groups for particular types of PAAEs at certain ages, there was no difference overall.

We agree that these figures are interesting and we now present them in the manuscript (page 12).

**Point 12:** The summary of Figure 2 presented in the text which states that at 24 months "the proportion of digestive PAAEs did not remain statistically decreased" is correct but obscures the fact that there remained a non-significant but noticeable decrease at this time point.

We have modified this sentence as follows: "*At 24 months, the proportion of digestive PAAEs remained decreased in the HKBBST group, as compared to the placebo group, but this did not reach significance (p=0.08).*"

**Point 13:** SCORAD - I am having difficulty seeing how this measure of eczema severity has been used in the analysis, despite its mention in the Methods section and the author's response to me. The cutaneous PAAEs include the presence of "atopic dermatitis" which is combined with other cutaneous PAAEs and reported as proportions in Figure 2. But where are the SCORAD (severity) results by study group, which I would expect to be reported as mean (SD) values? It is not clear that the statement "No significant difference was found with regards to intensity of PAAEs" (page 12) refers to the findings for SCORAD, but I would recommend that SCORAD results are presented separately as this is well recognized internationally accepted standard measure of eczema severity and it is important that these findings are explicit and visible. Furthermore, other studies of probiotics have reported the effect on SCORAD, and comparative data would be interesting.

**Point 14:** Severity of other PAAEs - The authors state (page 8) that each adverse event is scored between 0 and 4. A description of how these variables describing severity were analysed is not given in the Statistical analysis section, and no results appear to be reported.

Response to points 13 and 14:

We agree that these figures are of interest for the reader. We had removed them from the submitted manuscript because of limitation in the number of illustrations for publication in EJCN. We have now added these results to Table III. Nonetheless, because of the already high number and size of tables and figures, Table III could be available online only.

Actually, adverse events were scored minor, moderate or severe. There was an error in the writing page 8. It has now been corrected.

**Point 15:** Oral food challenge - As suggested above, a flow chart showing the decision making process for determining which children have which outcomes measured, including the oral food challenge, would be helpful. I'm also not sure why children with a negative OFC were given a placebo challenge. I would have expected that the children with a positive OFC would be the ones to placebo test to ensure that any response was greater than a response to a placebo.

As stated in our response to point 8, we now present a decision tree showing the tests performed. With regard to the second test performed in case of positive OFC, it was not a placebo test but tests against placebo. That is to say that both CM and placebo were blindly tested. We acknowledge that text allowed confusion, so that this is now made clearer in the method section. Also, as indicated above, we depict the place of "Home Food Challenge"

which followed a negative OFC.

**Point 16: Statistical analysis section - I appreciate the authors' elaboration of this section in response to my comments. However the details of specific symptoms are not necessary here. I would prefer the use of the term "continuous" rather than "quantitative" and "dichotomous" than "qualitative". However, the results for the skin prick tests and IgE level seem to have been defined only as dichotomous variables (positive vs negative) (Table II) - not as continuous variables as suggested by the authors in this section (although looking at mean differences between study groups is another possible analysis). Age, birth height, birth weight, breast feeding duration, and time for infant formula to become the main food are the only variables reported as continuous variables and (assuming they follow a normal distribution pattern) are appropriately reported as means and SD (Table I). It is also really important that the authors state in the manuscript that the reported analysis was per-protocol, and that the findings were similar when an intention-to-treat analysis was performed.**

We agree with the reviewer about classification of variables into quantitative or qualitative, and thank him for evidencing this error.

We do not entirely agree that “qualitative” can be replaced by “dichotomous” or “quantitative” by continuous. This is especially true for duration of feeding. When analyzed as a quantitative variable, it was not continuous but discreet (i.e. whole numbers of days only). When analyzed as qualitative variable, it was not dichotomous but comprised 3 modalities. This is the reason why we prefer the broader terms “quantitative” and “qualitative” variables.

In addition, we have now removed listing of the symptoms in this section.

Finally, the Statistical analyses section now includes a statement that presented analyses are from the PP population, and that these results showed no substantial difference as compared with ITT population.

**Point 17: Table II -**

**1. The footnote to the table now states that the row labelled as "Sensitization to cow's milk" (and the corresponding rows for "other foods" and "aeroallergens") is defined as a "positive skin test to this allergen or positive specific IgE". However total N for each of these sensitization groups suggests that a positive response to the patch test is also included in this definition. Please clarify.**

Yes, “skin test” included Skin Prick Tests and Atopy patch tests, which are thus included in the definition. A difference was seen only for milk SPTs

**Point 18: 2. The asterisks, which refer the reader to the definition in the footnote, is missing from the "other food" and "aeroallergen" groups.**

Thank you for underlining this error. We have now included asterisks where indicated.

**Point 19: 3. I notice that the sub-group analyses for skin prick tests, patch tests and IgE are not presented for aeroallergens. I expect this is because of the low numbers positive to aeroallergens, in which case the authors need to state this in the manuscript (or the footnote to the table).**

As stated in the Objectives section, “*The primary objective of this [...] study was to determine the effect of HKBBST milk on the incidence of CMA and CM sensitization in children at high risk of atopy.*” As a consequence, APT and IgE quantifications targeted food allergens (i.e. CM, hen’s egg, codfish, wheat flour, soy flour and roasted peanut). The search for sensitization to aeroallergens was only exploratory. This is the reason why, as indicated into

brackets in Table 2, sensitization to aeroallergens were searched using skin prick tests only (“*Sensitization\*\* to aeroallergens (SPT)*”). This decision is eventually supported by the very low number of sensitization to aeroallergens (Table II: n=13 during the whole study duration), as compared to sensitization to cow’s milk or to other foods, in this population.

**Point 20: Discussion -**

**The change in the 2nd para, page 13, is appropriate for this study design but the English could be improved. I suggest that the sentence end: ".....positive biological test or PAAE may have resulted in a decreased incidence and/or severity of CMA and sensitization.**

We have modified the sentence accordingly.

**Point 21: Could the authors be more explicit when referring to "biological tests" please?**

We have indicated the tests concerned at first occurrence of the expression “biological tests” in discussion: “*biological test (i.e., SPT, APT or IgE)*”.

**Point 22: On page 15, the new discussion around early symptoms that may or may not be allergic is appropriate although it could be reduced in length.**

We have slightly reduced this paragraph.

**Point 23: Also the authors do not actually present any data to show that the PAAEs correspond to objective symptoms (line 325-6).**

We agree with this comment, and that no measurement of objectivity of the symptoms was performed. Therefore, we have replaced “*objective*” by “*commonly observed in clinical practice*”.

**Point 24: The word "invalidating" is incorrectly used.**

We apologize for this Gallicism. “*Invalidating*” has now been replaced by “*incapacitating*”.

**Point 25: Line 332 - Replace "stop" with "cessation".**

This modification has been made.

1 **A non-hydrolyzed, fermented milk formula reduces digestive and**  
2 **respiratory events in infants at high risk of allergy**

3  
4  
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18  
19 **Running title:** Fermented formula and tolerance to cow's milk

20  
21 The study was supported by Blédina, France (Danone group).  
22  
23



## 23 **Abstract**

24 **Objective:** To determine the impact of a not hydrolyzed fermented infant formula containing  
25 heat-killed *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065 (HKBBST) on  
26 the incidence of allergy-like events during the first two years of life in children at high risk of  
27 atopy.

28 **Methods:** This multicenter, randomized, double-blind, controlled study included infants at  
29 high risk of atopy. Infants used HKBBST or a standard infant formula (SIF) since birth until 1  
30 year of age, and were followed at 4, 12 and 24 months after birth. Skin prick tests (SPT) for 6  
31 foods and 6 aeroallergens were systematically performed and adverse events (AE) were  
32 recorded. In case of potentially allergic AE (PAAE), allergy could be further tested by SPT,  
33 patch tests and quantification of specific IgEs. If cow's milk allergy (CMA) was suspected, an  
34 oral challenge could also be performed.

35 **Results:** The study included 129 children, 63 were randomized to SIF, 66 to HKBBST. The  
36 use of HKBBST milk did not alter the proportion of CMA but decreased the proportion of  
37 positive SPT to cow's milk (1.7% vs. 12.5%,  $p=0.03$ ), and the incidence of digestive (39% vs.  
38 63%,  $p=0.01$ ) and respiratory potentially allergic adverse events (PAAEs) (7% vs. 21%,  
39  $p=0.03$ ) at 12 months, and that of respiratory PAAEs at 24 months (13% vs. 35%,  $p=0.01$ ).

40 **Conclusion:** HKBBST decreased the incidence of PAAEs in children with family history of  
41 atopy, during the first months of life and after the formula was stopped. Oral tolerance to  
42 Cow's milk in infants at high risk of atopy may therefore be improved using not hydrolyzed  
43 fermented formulae.

44

45 **Key words:** Fermented infant formula; *Bifidobacterium breve*; *Streptococcus thermophilus*;  
46 prebiotic; cow's milk allergy; family history of atopy.

47

## 47 INTRODUCTION

48 Strategies currently proposed to decrease the incidence of cow's milk allergy (CMA) in at-  
49 risk children focus on the elimination of allergens: breastfeeding, delayed food  
50 diversification, elimination of allergenic foods and the use of formulae with hydrolyzed  
51 proteins. All these strategies are still controversial and their actual efficacy remains to be  
52 confirmed (Greer *et al*, 2008, Host *et al*, 2008, Osborn and Sinn, 2006, von Berg *et al*, 2007).

53 Based on the impact of intestinal microbiota modifications on food allergies, new dietetic  
54 strategies are currently being proposed, including the addition of prebiotics and probiotics to  
55 hydrolyzed infant formulae to prevent atopic diseases (Betsi *et al*, 2008, Boehm and Moro,  
56 2008, Giovannini *et al*, 2007, Isolauri *et al*, 2000, Kalliomaki *et al*, 2001, Kukkonen *et al*,  
57 2007, Rosenfeldt *et al*, 2003, Viljanen *et al*, 2005, Wold, 1998). Nonetheless, their efficacy  
58 and tolerance has still not been proven (AFSSA, 2003, Agostoni *et al*, 2004, Agostoni *et al*,  
59 2007, FAO/WHO, 2002, Hol *et al*, 2008, Kopp *et al*, 2008, Lee *et al*, 2008, Osborn and Sinn,  
60 2007).

61 Preclinical studies identified *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065  
62 as good candidates for the improvement of oral tolerance in children at high risk of atopy  
63 (Agostoni *et al*, 2007). Milk fermented with these two bacteria and heated to kill ferments has  
64 shown to modify intestinal microbiota in animals and humans (Romond *et al*, 1997, Romond  
65 *et al*, 1998, Thibault *et al*, 2004), to strengthen intestinal barrier in mice (Menard *et al*, 2006)  
66 and to prevent the increase of thymus size in non-breastfed, healthy term infants (Indrio *et al*,  
67 2007). Moreover, the metabolites of these ferments exert immunomodulatory properties,  
68 partly through dendritic cells (Hoarau *et al*, 2006, Menard *et al*, 2004). An infant formula  
69 made of milk fermented with *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065  
70 and heated to kill ferments (HKBBST) was therefore developed.

71 We report here the results of a controlled clinical trial of the efficacy of this not hydrolyzed  
72 fermented infant formula containing HKBBST on the incidence of CMA, sensitization to CM  
73 and potentially allergic adverse events (PAAE) during the first 24 months of life in children at  
74 high risk of atopy. In view of the early appearance of these events (Kvenshagen *et al*, 2008), a  
75 particular focus was made on the first 12 months of life.

76

## 76 **METHODS**

### 77 **Objectives**

78 The primary objective of this multicenter, randomized, double-blind, controlled study was to  
79 determine the effect of HKBBST milk on the incidence of CMA and CM sensitization in  
80 children at high risk of atopy.

81 Secondary objectives were to determine the effect of HKBBST milk on 1) the incidence of  
82 sensitization and/or allergy to other allergens than CM and 2) the incidence of allergic  
83 symptoms during the study period.

84

### 85 **Subjects**

86 Healthy male and female children presenting with family history of allergy at one of the two  
87 centers (Neonatology Unit, Saint-Vincent de Paul Hospital, Paris, France or Unit of Internal  
88 Medicine, Clinical Immunology and Allergology, Nancy University Hospital, Nancy, France)  
89 or at one of the 16 private allergologist investigators were included. Children were  
90 randomized in sequential ascending order to either standard infant formula or HKBBST milk.

91 The randomization list was created by an independent statistician (OPTIMED, France) and  
92 confidentially maintained until approval was received for the study to be unblinded for  
93 analysis. Randomization occurred after the selection (i.e. before the end of the 5<sup>th</sup> month of  
94 pregnancy). To control possible breastfeeding effect, women wishing to breastfeed were  
95 randomly balanced between groups. Sample size was calculated based on the primary  
96 outcome. The mean percentage of at-risk children who effectively develop allergic  
97 manifestations is fairly imprecise, between 6% and 20%, the risk being approximately divided  
98 by two by probiotics (Exl and Fritsche, 2001, Kalliomaki *et al*, 2001, Saarinen *et al*, 1999).

99 Based on these data, for rejection of two-sided null hypothesis with a type I error of 5% and a  
100 type II error of 30%, at least 62 children had to be included per group, for a total of 124

101 children. Prior to any action under the study, the investigator collected written consent from  
102 both parents or from the legal representative. The protocol was approved by the French ethics  
103 committee and the study was conducted in accordance with the Declaration of Helsinki as  
104 (revised in 2004), the regulatory provisions applicable in France and Good Clinical Practices.  
105 The study was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) under the reference NCT00792090.

106

### 107 **Inclusion criteria**

108 Inclusion criteria were less than six months of pregnancy and family at risk of atopy: at least  
109 two atopic members, including at least one of the parents (i.e. mother and/or father and/or at  
110 least one brother or sister). Children with known or suspected immune deficiency within  
111 family were not included. In addition, because actual use of the formula could not be  
112 determined in children without known date of weaning, they were excluded from analyses.

113

### 114 **Interventions**

115 Selection included examination by an allergist and interview with a dietician. Medical and  
116 diet questionnaires were filled-in and mothers were explained the diet to be followed until the  
117 fourth month after birth and were given advice to prevent atopy. Mothers wishing to  
118 breastfeed were encouraged to do so up to the sixth month after birth. From the fifth month of  
119 pregnancy, the mother was required to use of calcium supplementation and avoid peanut, nut,  
120 egg, and fermented milk products or prebiotic- or probiotic-containing food (commercially  
121 available milk was allowed). Actual inclusion was at child's birth without additional visit.  
122 Parents then had to complete a diary collecting data on weaning and use of milk or formula.  
123 Every day, from birth to the end of breastfeeding, mothers of exclusively breastfed children  
124 had to drink at least 500 mL of the milk their child had been randomized to. The dietician  
125 checked diet compliance and tolerance by the mothers. Following visits were at 4 months

126 after birth ( $\pm 2$  weeks), 12 months ( $\pm 2$  weeks) and 24 months ( $\pm 4$  weeks). During visits, the  
127 allergist examined the child, filled out a demographic and medical questionnaire, collected  
128 adverse events (AEs) and PAAEs, and systematically performed skin prick tests (SPT). The  
129 allergist was free to perform atopy patch test (APT) or specific IgE quantification according  
130 to standardized procedures in case of allergy suspicion. The dietician explained the diet to be  
131 followed during the following months (gradual diversification) and filled out a dietetic  
132 questionnaire. At the end of breastfeeding, children gradually moved to the formula they had  
133 been randomized to. Partially breastfed children were given from birth the formula they had  
134 been allocated to. The formula had to be used until the age of one year. Parents visited the  
135 allergist each time a clinical event appeared.

136

### 137 **Products under study**

138 Control was a standard infant formula (Gallia<sup>®</sup>, Blédina, France). HKBBST formula had the  
139 same nutritional composition as the standard infant formula but was fermented with  
140 *Bifidobacterium breve* C50 ( $4.2 \times 10^9$  bacteria per 100g of powder formula) and *Streptococcus*  
141 *thermophilus* 065 ( $3.84 \times 10^7$  bacteria per 100g of powder formula) during the manufacturing  
142 process and then heated to kill ferments (patented technology, Blédina, France). The  
143 appearance, taste and smell of the two products were similar. Allergist, dietitian and mother  
144 were blinded to the product allocated during all the study, until the analysis of last result data.

145

### 146 **Outcomes measurement**

147 Children were considered allergic to CM and/or to another food allergen when Oral Food  
148 Challenge (OFC) was positive. Children were considered sensitized to a food allergen in case  
149 of positive skin tests (SPT or APT) or positive specific IgE, independently of allergy-like  
150 symptoms.

151

**152 Data collected and tests performed (Fig. 1)****153 PAAEs**

154 OFC being performed after 6 months of age only, physicians were asked to collect AEs and  
155 identify those that have been shown to be associated with the development of allergy during  
156 the first months of life (potentially allergic adverse events – PAAEs) (Host *et al*, 2008,  
157 Kvenshagen *et al*, 2008, Vandenplas *et al*, 2007). PAAEs were grouped into three categories:  
158 i) cutaneous symptoms included atopic dermatitis, urticaria, angioedema, eczema, dry skin  
159 when associated to another symptom, rash, and pruritus, ii) digestive symptoms included  
160 upper digestive symptoms (vomiting and gastro-esophageal reflux), lower digestive  
161 symptoms (acute diarrhea, colitis, abdominal pain, constipation, abdominal distension, rectal  
162 bleeding) and general symptoms associated (fainting or transient hypotonia and failure to  
163 thrive), and iii) respiratory symptoms included wheezing, wheezing bronchitis and spastic  
164 bronchitis. The severity of digestive symptoms was indirectly estimated using the growth  
165 curve, with occurrence of a failure to thrive. Each adverse event has been annotated by the  
166 allergist and scored minor, moderate or severe.

167

**168 Skin prick test**

169 SPTs were standardized and systematically performed at each visit. They were made  
170 according to the prick-in-prick method (Dreborg, 1991). SPT to food allergens included CM,  
171 hen's egg, codfish, wheat flour, soy flour and roasted peanut. SPT to aeroallergen extracts  
172 included *Dermatophagoïdes pteronyssinus*, cat and dog dander, mix of grass pollens, birch  
173 pollen and *Alternaria alternata* (Allerbio Laboratories, Varennes-en-Argonne, France). A  
174 positive control (9% codeine phosphate) and a negative control (solvent, *i.e.* saline) were

175 systematically performed. SPT was considered positive if the mean wheal diameter was more  
176 than 75% of the positive control wheal or greater than or equal to 3 mm.

177

### 178 **Atopy patch test**

179 APTs were standardized and performed using a Finn chamber occluded during 48 hours,  
180 according to the method described by Kekki *et al.* (1997). APTs were performed with CM,  
181 hen's egg, codfish, wheat flour, soy flour and roasted peanut. Reading was made at 72h.

182 APT was considered positive according the International Contact Dermatitis Research Group  
183 (ICDRG) and the European Task Force on Atopic Dermatitis (ETFAD) Consensus Meetings  
184 criteria (Turjanmaa *et al.*, 2006).

185

### 186 **IgE detection**

187 Blood was sampled when an allergic was suspected. Specific CM or food allergen IgEs were  
188 quantified by ImmunoCap<sup>®</sup> (Phadia, Uppsala, Sweden) and considered as positive for scores  
189  $\geq 0.35$ kUI/L.

190

### 191 **OFC**

192 Children with CMA suspicion were submitted to an OFC to CM after two months of an  
193 elimination diet and disappearance of the symptoms. If the OFC was negative or doubtful, a  
194 second, double-blind OFC was performed by testing CM against placebo (Neocate<sup>®</sup>,  
195 Nutricia).

196 Criteria considered for positive OFC to CM were onset, reappearance or worsening eczema  
197 lesions, urticaria, angioedema, anaphylactic shock, hemodynamic modification, fainting,  
198 cough, dyspnea, wheezing, diarrhea, abdominal pain, vomiting and constipation. According to  
199 Garcia-Ara *et al.* (2001), who previously showed a positive predictive value of CM allergy



200 >95% for children less than 12 months when specific IgE level are greater than 5 kU/l. OFC  
201 was not performed if SPT to CM was positive *and* specific IgEs were >5kUI/L. For ethical  
202 considerations, oral challenge was not performed in case of previous history of anaphylactic  
203 shock, laryngeal edema, severe asthma, or any life-threatening immuno-allergic complaint.  
204 Since food allergy manifestations with digestive symptoms can be >72 hours, OFC was  
205 sometimes followed by home food challenge. OFC was performed as early as possible but  
206 never before the age of six months.

207

### 208 **Statistical analyses**

209 Tests were two-sided and the level of significance ( $\alpha$ ) was set at 0.05. Chi<sup>2</sup> and Fisher's exact  
210 tests were used for qualitative variables (test: positive or negative / symptom observed: yes or  
211 no): skin or IgE testing, cutaneous, digestive, general and respiratory symptoms. Results  
212 presented here are from Per-Protocol populations, which showed no substantial difference as  
213 compared with ITT populations. Statistical analyses were performed using SAS, version 8.2  
214 (SAS Institute, North Carolina).

215

## 215 **RESULTS**

216 From January 2004 to April 2006, 136 mothers were selected and 129 children were included  
217 and randomly allocated to standard infant formula (n=63, of which 55 completed the study) or  
218 HKBBST milk (n=66, of which 53 completed the study) (Fig. 2). Characteristics of the  
219 children at inclusion are described in Table 1 and no statistically significant difference was  
220 observed between groups. Use of other milk or formula during the study was low and did not  
221 differ between groups.

222

### 223 **Sensitization and CMA**

224 At 4 months, a trend was observed towards a lower proportion of positive SPT to CM in the  
225 HKBBST group than in the standard group (1.6% vs. 7.9%; p=0.06) (Table II). However, no  
226 significant difference was found between groups concerning sensitization to CM, other foods  
227 or aeroallergens. OFC was not performed before the age of 6 months, so that CMA could not  
228 be diagnosed at 4 months.

229 At 12 months, although CMA incidence was not different between groups, the proportion of  
230 positive SPT to CM was significantly decreased in the HKBBST group as compared to the  
231 standard group (1.7% vs. 12.5%; p=0.03) (Table II). When specific IgEs for foods other than  
232 CM were quantified, children in the HKBBST group showed a higher proportion of negative  
233 biological test than children in the standard group (90.9% vs. 33.3%; p=0.01). APT to CM,  
234 CM-specific IgEs, SPT and APT to other foods and sensitization to aeroallergens were not  
235 different between groups.

236 The incidence of CMA remained not different between groups at 24 months but the  
237 proportions of positive SPT to CM and children with detectable anti-milk IgE tended to be  
238 decreased in the HKBBST group as compared to the standard group (Table II). No difference  
239 was observed between groups with regards to sensitization to other foods.

240

241 **PAAEs**

242 During the study, 43 of the 55 children (78.2%) had at least 1 PAAE in the standard group;  
243 they were 37 of the 53 children of the HKBBST group (69.8%) ( $p=0.32$ ). Compared to  
244 standard infant formula, HKBBST milk significantly decreased the proportion of children  
245 with at least one digestive PAAE at 4 months (42.9% vs. 26.2%;  $p=0.047$ ) (Fig. 3). At 12  
246 months, children of the HKBBST group showed a significantly lower proportion of  
247 respiratory PAAEs (21.4% vs. 6.8%;  $p=0.031$ ) and digestive PAAEs (62.5% vs. 39.0%;  
248  $p=0.012$ ), as compared to standard group. At 24 months, the proportion of digestive PAAEs  
249 remained decreased in the HKBBST group, as compared to the placebo group, but this did not  
250 reach significance ( $p=0.08$ ). However, the decreased proportion of respiratory PAAE  
251 observed at 12 months in the HKBBST group remained significant at 24 months (34.6% vs.  
252 13.2%,  $p=0.010$ ). No significant difference was found with regards to general intensity of  
253 PAAEs (Table III).

254

255

## 255 **DISCUSSION**

256 The present study did not show decreased incidence of CMA in children using HKBBST  
257 milk. However, HKBBST milk decreased the proportion of positive SPT to CM, the  
258 incidence of digestive and respiratory PAAEs, and the proportion of positive IgE tests against  
259 other foods than CM over the first 12 months of life. In addition, the impact of HKBBST  
260 persisted until the 24<sup>th</sup> month of life with decreased incidence of respiratory PAAE. As such,  
261 this is the first study showing efficacy of a fermented infant formula with non-hydrolyzed  
262 milk proteins on respiratory and digestive allergic manifestations and biological signs of  
263 sensitization to food during the first year of life.

264

265 The present study was performed on a subsample of the general population (*i.e.* children at  
266 high risk of atopy) but the proportion of CMA was lower than expected. Nonetheless, the  
267 rapid and careful follow up by the dietician and the allergist of children presenting positive  
268 biological test (*i.e.*, SPT, APT or IgE) or PAAEs may have resulted in a decreased incidence  
269 and/or severity of CMA and sensitizations cannot be ruled out.

270 With regard to biological tests and PAAEs, the trend observed at 4 months towards a lower  
271 proportion of positive SPT to CM became significant at 12 months and lasted until the 24<sup>th</sup>  
272 month of life, well after HKBBST had been stopped. In addition, the proportion of children  
273 with digestive PAAEs at 4 months was almost twice as low in the HKBBST group as in the  
274 standard group, which persisted until at least the 12<sup>th</sup> month. With regards to respiratory  
275 PAAEs, a trend towards lower incidence could be observed at 4 months that increased during  
276 the study to be significant at 12 and 24 months. This study is the first to show the early and  
277 persisting protective effect of a fermented infant formula without live bacteria, and containing  
278 intact, not hydrolyzed CM proteins, on biological tests and PAAEs in infants with family  
279 history of atopy. Previous preclinical and clinical studies suggest that this may be related to

280 the positive effect of HKBBST milk on intestinal flora and thymus size in healthy infants  
281 (Indrio *et al*, 2007, Thibault *et al*, 2004).

282 The particularity of HKBBST milk resides in its original formulation. It is neither a  
283 hypoallergenic formulation, in which casein would have been hydrolyzed, nor a formulation  
284 exclusively containing probiotics or prebiotics. In addition, HKBBST formula was the same  
285 formula as the standard infant formula used as comparator in the present study, but fermented  
286 with *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065 and heated to kill  
287 ferments. Both milks were not hydrolyzed and contained intact CM proteins, which was  
288 confirmed by gel electrophoresis and liquid chromatography (data not shown). Therefore, the  
289 effect we describe can be attributed to the fermentation with *Bifidobacterium breve* C50 and  
290 *Streptococcus thermophilus* 065 followed by heating. As suggested by the study by Sashihara  
291 *et al.* (2006) showing in a murine model of allergy that proteoglycans derived from different  
292 heated-killed strains of lactic bacteria modulate cytokines production, this effect may be due  
293 to thermoresistant peptidoglycans contained in the cell wall of the ferments used. The role of  
294 peptidoglycans or of other components of the cell wall is further supported by the fact that  
295 *Bifidobacterium breve* C50 can activate the Toll-like receptor (TLR)2 (Hoarau *et al*, 2006),  
296 the main ligands of which are components of the cell wall of Gram-positive bacteria.

297

298 Comparison of our results with similar infant formulae (*i.e.* prebiotic- or probiotic-containing  
299 formulations) is only possible to a very limited extent since very few studies have been  
300 published that concerned a not hydrolyzed formula in children under one year of age and at  
301 increased risk of allergy (Betsi *et al*, 2008). Nonetheless, our results support the study by  
302 Kalliomaki *et al.* (2001), which showed that the use of probiotics could prevent allergy-like  
303 events (*i.e.*, a 50% decrease in the incidence of atopic dermatitis) in children under one year.  
304 However, the study in question showed no impact on the proportion of food sensitizations and

305 its results failed to be replicated (Kopp *et al*, 2008). Our results also support the study of  
306 Kukkonen *et al*. (2007), which has shown that the use of a preparation containing 4 different  
307 probiotics along with galacto-oligosaccharides prevented eczema, but this was shown in two  
308 years old children. With regard to other infant formulae, even if efficacy has still not been  
309 proven for soybean protein-based formulae, some formulae with partially or completely  
310 hydrolyzed casein (hypoallergenic) have shown good results. However, due to differences in  
311 methodologies and results, generalization across studies is difficult and no consensus was  
312 found so far concerning their use (Greer *et al*, 2008).

313 Comparison with other studies is also difficult because of the very young age of the children  
314 at inclusion and the particular methodology we had to design. Because the diagnosis of an  
315 allergy can hardly be set before 6 months of age, any symptom (*i.e.* PAAEs) or biological  
316 data possibly related to allergy had to be collected, whereas most studies mainly collect and  
317 analyze atopic dermatitis. PAAEs were clinical symptoms that could correspond to allergic  
318 symptoms but for which clear association with allergy could not be made. Established rule  
319 states that the allergenic origin of a symptom is confirmed only if it disappears after allergen  
320 avoidance and reappears during OFC. For some children who presented with PAAEs  
321 disappearing after CM eviction, it was not possible to prove the role of CM because the oral  
322 challenge was negative. One can wonder whether these symptoms were, or not, transient  
323 symptoms of CM allergy with rapid natural recovery before the age of 6 months. We are fully  
324 aware that a positive biological test or PAAEs are not sufficient to set the diagnosis of an  
325 allergy. Nonetheless, even if the PAAEs considered are not as solid as OFC, they correspond  
326 to symptoms commonly observed in clinical practice, which are most often incapacitating for  
327 children and families; their avoidance would largely improve the quality of life of the children  
328 and their families. Furthermore, this study demonstrates that studies on the impact of an infant  
329 formula on allergy-like events can be conducted within the first year of life.

330

331 In conclusion, the present study shows that the HKBBST fermented infant formula,  
332 containing intact milk proteins, decreases the proportion of sensitization to CM proteins and  
333 the incidence of PAAEs in children at high risk of atopy, as early as the very first months of  
334 life and during a long period of time after its cessation. Fermented milks may represent a new  
335 dietetic strategy to promote oral tolerance to CM.

336

337

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344

### 345 **Conflict of interest**

346 Cécile Aubert-Jacquin, is Danone employee.

347 Martine Morisset, Pascale Soulaines, Denise-Anne Moneret-Vautrin and Christophe Dupont  
348 have received honoraria and/or compensation in regards to the study, as an investigator,  
349 coordinator or expert, in relation with the time spent on the study. The authors declare no  
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351

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452

453

453 **TABLE I.** Characteristics of the children included in the study.

	<b>Total</b> <b>N=129</b>	<b>Standard</b> <b>N=63</b>	<b>HKBBST</b> <b>N=66</b>	<b>p-value</b>
Gestational age (weeks)	39.1 ± 1.2	39.0 ± 1.2	39.3 ± 1.2	0.18
Birth weight (kg)	3.39 ± 0.39	3.32 ± 0.35	3.46 ± 0.42	0.053
Birth height (cm)	50.1 ± 2.1	49.8 ± 2.1	50.4 ± 2.1	0.13
Female gender	58 (45.0)	31 (49.2)	27 (40.9)	0.34
Family history of allergy				
Mother	95 (73.6)	44 (69.8)	51 (77.3)	0.34
At least one of mother's offspring	116 (89.9)	56 (88.9)	60 (90.9)	0.7
Father	105 (81.4)	55 (87.3)	50 (75.8)	0.09
At least one of father's offspring	111 (86.0)	54 (85.7)	57 (86.4)	0.91
Duration of breastfeeding (days)	113.3 ± 91.6	122.6 ± 106.8	103.5 ± 72.3	0.66
No breast feeding	47 (36.4)	21 (33.3)	26 (39.4)	
]0-4 months]	46 (35.7)	25 (39.7)	21 (31.8)	
> 4 months	36 (27.9)	17 (26.9)	19 (28.8)	
Time (days) for infant formula to become the main food	126.2 ± 73.2	116.5 ± 71.8	135.1 ± 74.2	0.24

454 *Results are presented as mean ± SD or number (percent).*

455 **TABLE II.** Allergy and/or sensitization to cow's milk, other foods and aeroallergens at 4, 12 and 24 months,

	4 MONTHS				12 MONTHS				24 MONTHS			
	Total	Standard	HKBBST	<i>p</i>	Total	Standard	HKBBST	<i>p</i>	Total	Standard	HKBBST	<i>p</i>
	N=128	N=63	N=65		N=115	N=56	N=59		N=108	N=55	N=53	
<b>Cow's milk allergy*</b>	na	na	na	na	15 (31)	9 (15)	6 (16)	0.64	28 (30)	15 (16)	13 (14)	1.0
<b>Sensitization** to cow's milk</b>	23 (125)	14 (63)	9 (62)	0.11	32 (115)	18 (56)	14 (59)	0.31	34 (108)	20 (55)	14 (53)	0.27
<i>Skin Prick Test</i>	6 (125)	5 (63)	1 (62)	0.06	8 (115)	7 (56)	1 (59)	0.03	8 (108)	7 (55)	1 (53)	0.06
<i>Atopy patch test</i>	21 (45)	12 (22)	9 (23)	0.53	27 (52)	15 (24)	12 (28)	0.36	28 (53)	17 (26)	11 (27)	0.16
IgE $\geq 0.35$ kUI/L	3 (31)	1 (15)	2 (16)	1.0	9 (46)	7 (22)	2 (24)	0.13	11 (45)	8 (22)	3 (23)	0.07
<b>Sensitization** to other foods</b>	29 (126)	13 (63)	16 (63)	0.20	44 (115)	21 (56)	23 (59)	NS	45 (108)	22 (55)	23 (53)	0.72
<i>Skin prick test</i>	8 (126)	3 (63)	5 (63)	0.19	24 (115)	11 (56)	13 (59)	0.75	25 (108)	12 (55)	13 (53)	0.74
<i>Atopy patch test</i>	26 (46)	13 (23)	13 (23)	0.98	35 (54)	19 (26)	16 (28)	0.48	35 (53)	20 (26)	15 (27)	0.14
IgE $\geq 0.35$ kUI/L	0 (5)	0 (1)	0 (4)	0.36	3 (14)	2 (3)	1 (11)	0.01	5 (17)	3 (6)	2 (11)	0.28
<b>Sensitization** to aeroallergens (SPT)</b>	2 (125)	1 (63)	1 (62)	0.24	7 (115)	4 (56)	3 (59)	0.71	13 (108)	6 (55)	7 (53)	0.71

456 *Results are presented as n (number of children tested). \* Allergy was confirmed by a positive Oral Food Challenge (OFC) with immediate or delayed reaction; OFC was not*  
457 *performed before 6 months of age. \*\* Sensitization to an allergen corresponded to positive skin test(s) to this allergen or positive specific IgE (ImmunoCap Phadia®*  
458 *>0,35kU/l). SPT was considered positive if the mean wheal diameter was  $\geq 75\%$  of the positive control wheal or  $\geq 3$  mm. APT was considered positive according to the*  
459 *International Contact Dermatitis Research Group (ICDRG) and the European Task Force on Atopic Dermatitis (ETFAD) Consensus Meetings criteria (Turjanmaa et al,*  
460 *2006).Skin Prick Tests were systematically performed; other tests were decided by the investigator. na, not applicable.*

461 **TABLE III.** Total number and severity of PAAEs at 4, 12 and 24 months.

	<b>4 MONTHS</b>				<b>12 MONTHS</b>				<b>24 MONTHS</b>			
	<b>Total</b> <b>N=128</b>	<b>Standard</b> <b>N=63</b>	<b>HKBBST</b> <b>N=65</b>	<b><i>p</i></b>	<b>Total</b> <b>N=115</b>	<b>Standard</b> <b>N=56</b>	<b>HKBBST</b> <b>N=59</b>	<b><i>p</i></b>	<b>Total</b> <b>N=108</b>	<b>Standard</b> <b>N=55</b>	<b>HKBBST</b> <b>N=53</b>	<b><i>p</i></b>
<b>Number of PAAEs</b>	72 (56.3)	38 (60.3)	34 (52.3)	0.36	81 (70.4)	42 (75.0)	39 (66.1)	0.29	80 (74.1)	43 (78.2)	37 (69.8)	0.32
<b>Intensity of PAAEs</b>				0.27				0.63				0.52
Minor	35 (48.6)	22 (57.9)	13 (38.2)		33 (40.7)	19 (45.2)	14 (35.9)		30 (37.5)	17 (39.5)	13 (35.1)	
Moderate	31 (43.1)	13 (34.2)	18 (52.9)		41 (50.6)	19 (45.2)	22 (56.4)		43 (53.8)	21 (48.8)	22 (59.5)	
Severe	6 (8.3)	3 (7.9)	3 (8.8)		7 (8.6)	4 (9.5)	3 (7.7)		7 (8.8)	5 (11.6)	2 (5.4)	
<b>Incidence of atopic dermatitis</b>	45 (35.2)	21 (33.3)	24 (36.9)	0.67	55 (47.8)	26 (46.4)	29 (49.1)	0.77	48 (44.4)	24 (43.6)	24 (45.3)	0.86
<b>Severity of atopic dermatitis</b>				0.27				0.77				0.72
Minor	24 (51.1)	14 (63.6)	10 (40)		38 (63.3)	18 (66.7)	20 (60.6)		51 (63)	25 (61)	26 (65)	
Moderate	19 (40.4)	7 (31.8)	12 (48)		18 (30)	8 (29.6)	10 (30.3)		27 (33.3)	15 (36.6)	12 (30)	
Severe	4 (8.5)	1 (4.5)	3 (12)		4 (6.7)	1 (3.7)	3 (9.1)		3 (3.7)	2.44 (3)	2 (5)	

462 *Results are presented as n (percentage).*

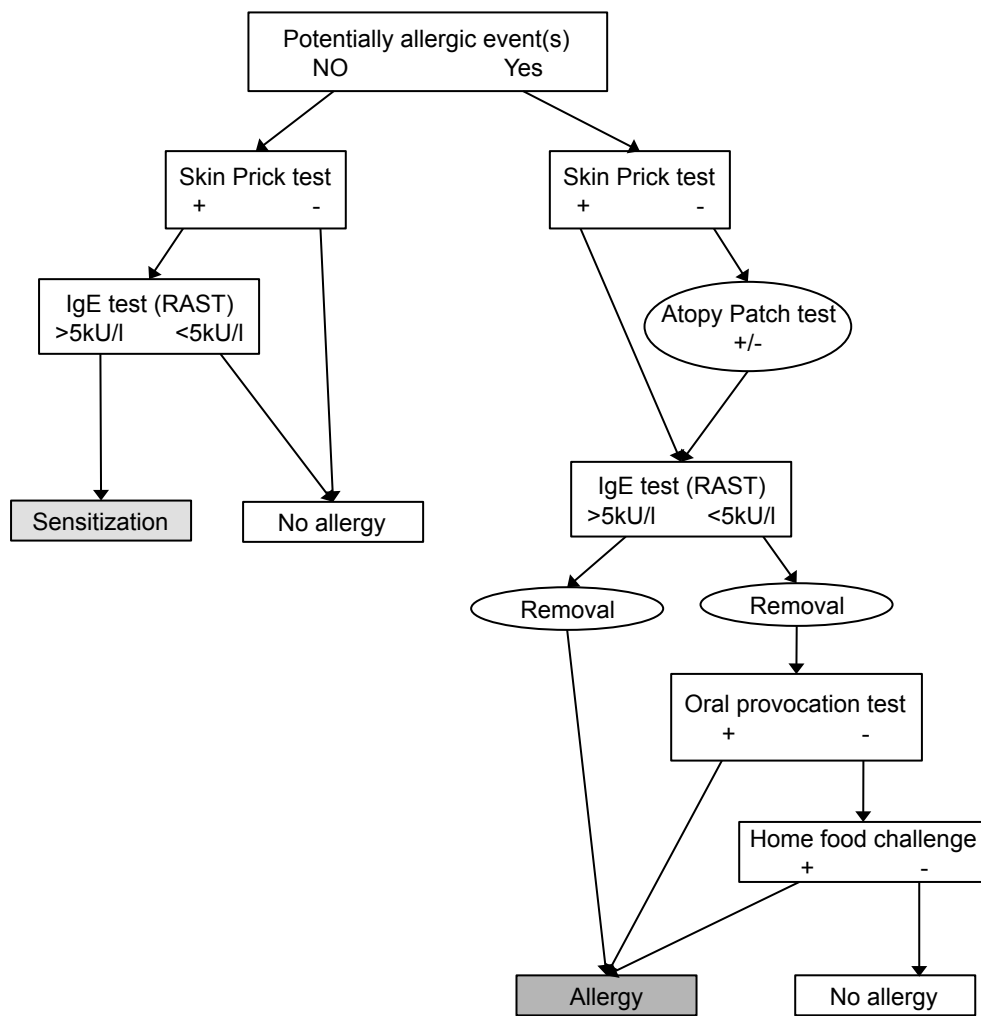
## Figure Legends

**FIG 1.** Decision tree of the tests performed during study. IgE quantification and atopy patch tests were performed only for CM and food allergens.

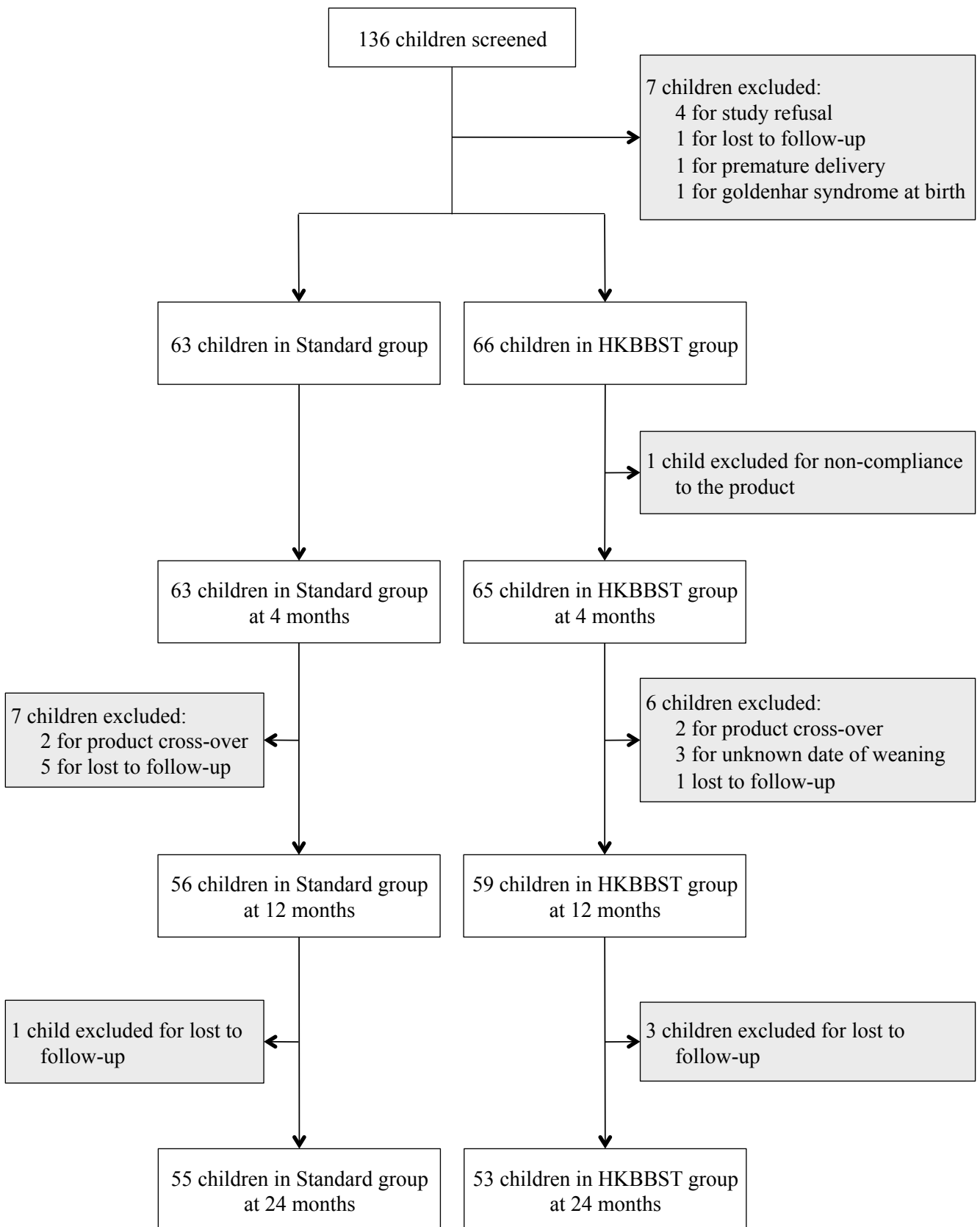
**FIG 2.** Flow diagram of the children participating in the study.

**FIG 3.** Percentage of children with respiratory, digestive or cutaneous PAAEs in standard and HKBBST groups at 4, 12 and 24 months.

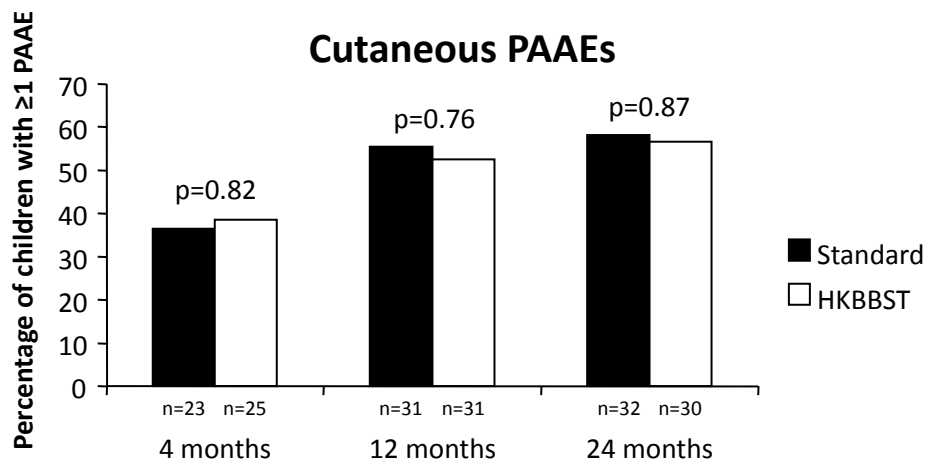
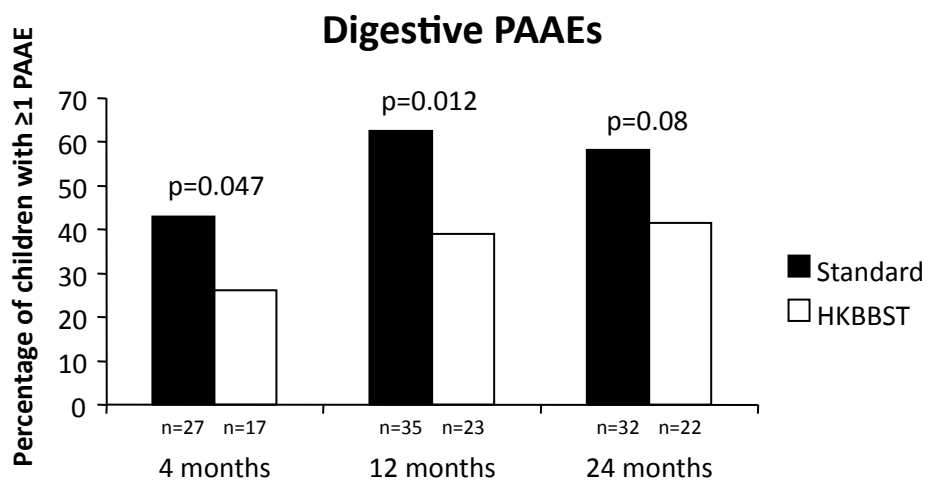
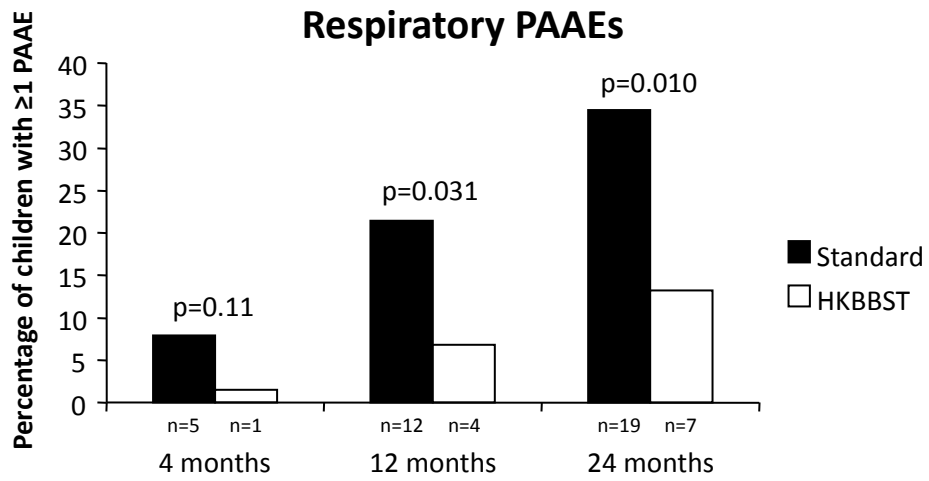




**Figure 1**



**Figure 2**



**Figure 3**