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A multi-center assessment to compare residual allergenicity of partial hydrolyzed whey proteins in a murine model for cow's milk allergy – Comparison to the single parameter guinea pig model

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ABSTRACT

Introduction: This 4-center study is part of a project to validate a food allergy murine model for safety testing of hydrolyzed infant formulas.

Aim: The aim of the current multi-center experiment was to evaluate the residual allergenicity of three partial hydrolyzed whey proteins (pWH) in a multiple-parameter cow's milk allergy murine model and to compare to the classically used guinea pig model. Previous work showed differences in the magnitude of the allergic response to whey between centers. To get a first insight in the effect of housing on the robustness of the mouse model, microbiota composition of non-sensitized mice was analyzed and compared between centers.

Methods: Mice were sensitized intragastrically (i.g.) with whey, pWH or eWH using cholera toxin as an adjuvant. In mice, whey-IgE/IgG1, acute allergic symptoms were determined upon whey challenge. Guinea pigs were orally sensitized ad libitum via the drinking water (day 0–37) and challenged intravenously with whey on day 49. The microbial composition in fecal samples was determined in non-sensitized mice in all 4 research centers before and after conduct of the study.

Results: Elevated levels of whey-IgG1 were detected in whey-sensitized mice in all centers. Except for pWH-A in center 4, we observed elevated levels of whey-IgE in whey-sensitized mice and mice sensitized with pWH-A, -B, -C. Center 2 was excluded from further analysis because of non-significant IgE levels in the positive control. In contrast to whey-mice, pWH-A treated mice showed no acute skin response, mMCP-1 release or change in body temperature upon whey challenge in all centers, which corresponds with the absence of anaphylactic shock symptoms in both the mouse and guinea pig model. pWH-B and -C induced anaphylactic shock symptoms in the guinea-pig and mice whereas results on the remaining allergic outcomes in mice were inconclusive. No differences in microbiota composition were measured in response to the challenge and Microbiota composition depended on the location of the centers.

Conclusions: Both animal models showed comparable results on the residual allergenicity of partial hydrolyzed whey proteins, but none of the centers was able to differentiate between the residual sensitizing capacities of the pWH-B and -C based on a single elicitation parameter in the murine model. Differences in microbiota composition might contribute to the robustness of the food allergy murine model. For a well-balanced prediction on the potential allergenicity of hydrolyzed infant formulas a multiple murine parameter model is suggested to decrease the risk of false positive or false negative results. A future challenge is to develop an overall scoring system for proper risk assessment, taking all parameters into account.

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1. Introduction

Milk proteins belong with proteins of peanut, tree nuts, fish, shellfish and hen's egg to the major allergens related to food-induced allergic responses (Sicherer and Sampson, 2018). Treatment strategies, like allergen-specific immunotherapy are upcoming promising approaches for the dietary management of food allergy, but still, these are often accompanied with high incidence of adverse allergic reactions during treatment, especially in severe cow's milk-allergic patients (Vonk et al., 2017b; Pajno et al., 2018). Consequently, current guidelines are usually based on allergen avoidance to prevent clinical symptoms. Hydrolyzed proteins are processed by enzymatic or heat treatment and/or by ultrafiltration of cow's milk proteins (whey or casein), and lack B-cell epitopes responsible for the IgE mediated allergic response, therefore, hydrolyzed cow's milk proteins are in principle unable to elicit allergic adverse events and are commonly used as infant nutrition for genetically predisposed infants or infants diagnosed with cow's milk allergy. These hydrolyzed formulas are generally categorized as partial and extensive infant formulas based on the degree of hydrolysis and can be characterized by assessing the molecular weight distribution of the residual proteins. In general, extensively hydrolyzed formulas contain fragments with a lower molecular weight distribution. For safe use in infants it is mandatory to assess the residual allergenicity of hydrolyzed proteins as used in infant formulas by showing that the hydrolyzed proteins are not able to sensitize animals to the protein source they are derived from ('Commission Directive 96/4/EC of 16th February, 1996 amending Directive 91/321/EEC on infant formulas and follow-on formulas.'). If the tested infant formulas are safe, animals should not respond with allergic symptoms to a challenge with intact protein.

We initiated several studies using a multiple-parameter murine IgE-mediated food allergy model to assess the potential allergenicity of hydrolyzed whey-based infant formulas (van Esch et al., 2011v). In a first phase study to standardize the cow's milk allergy mouse model, we have shown the lab-transferability of the model in as well as its ability to discriminate between the sensitizing properties of native whey protein and eWH (van Esch et al., 2013). Even though genetic background and specific conditions like age, breeder, diet, group size, bedding were standardized we observed low levels of allergen-specific IgE and IgG1 in one of the four centers (van Esch et al., 2013). We hypothesize that alterations in the microbial community due to changed housing of the mice from breeder to the different centers might have an impact on the robustness of the mouse model.

In the current study, we assessed the potency of the cow's milk allergy murine model to predict the allergenicity of three pWH in four independent research centers. Since historically, hypo-allergenicity studies are performed in male guinea pigs (Devey et al., 1976), we compared the results of the murine model with the anaphylactic shock guinea pig model. The microbial composition in fecal samples was determined in non-sensitized mice before and after conduct of the study.

2. Material and methods

2.1. Participating institutes

The multicenter ring trial was performed independently at four research centers in the Netherlands: the Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, Utrecht; TNO, Zeist; Nutricia Research, Utrecht; and the Utrecht Institute for Pharmaceutical Sciences (UIPS), Faculty of Science, Utrecht University, Utrecht. The guinea pig model was only performed at TNO Zeist, the Netherlands.

2.2. Test materials

Whey protein concentrate 80 (indicated as whey) was obtained from DMV International (Veghel, the Netherlands). The partial whey hydrolysate pWH-A, -B and -C were manufactured by enzymatic hydrolysis under specified conditions. The enzymatic process was stopped by fast cooling. The pWH's were further characterized by analysis of the molecular weight distribution. (**pWH-A**: 56,5% < 1 kDa, 10,8% < 2 kDa, 11,3 < 5 kDa, 6,5% < 10 kDa, 14,8% > 10 kDa), (**pWH-B**: 56,6% < 1 kDa, 11,4,8% < 2 kDa, 11,7% < 5 kDa, 6,7% < 10 kDa, 13,7% > 10 kDa) and (**pWH-C**: 53,3% < 1 kDa, 11,0% < 2 kDa, 12,0 < 5 kDa, 8,6% < 10 kDa, 15,1% > 10 kDa) by means of high pressure liquid chromatography. The extensively hydrolyzed protein (eWH) was hydrolyzed with an established mixture of endopeptidases and exopeptidases and ultra-filtrated (confidential enzyme composition used by Danone) resulting in an eWH (< 3KDa). This eWH is an experimental hydrolyzate solely produced for these experiments and not for usage in an end product. Three pWH's; pWH-A, pWH-B and pWH-C were kindly provided by FrieslandCampina Domo. All participating centers used whey, pWH and eWH (containing less than 0.01% of native protein) from the same batches.

2.3. Experimental setup murine model

Four to five week-old specific pathogen-free female C3H/HeOuJ mice (Charles River Centers, Saint Germain sur l'Arbresle, France) were used in all studies. The animals were raised and bred on a milk-free diet for at least two generations. Food and water were available ad libitum. The animals were maintained on semi-purified cow's milk protein-free mouse chow (AIN-93G-soja, Research Diet Services, Wijk bij Duurstede, The Netherlands). The ambient temperature was maintained between 20 and 24 °C and relative humidity was maintained between 45 and 65% with a 12 h light/dark cycle. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments (DEC 2011.III.08.89) and all experiments complied with the arrive guidelines. The mouse studies were conducted simultaneously in the 4 research centers. Therefore, all mice used were obtained from the same batch of nests, after which they were distributed randomly over the 4 research centers prior to the start of studies. At each center, mice were randomized over six groups, allowed to acclimatize for one week and then i.g. sensitized using a blunt needle on days 0, 7, 14, 21 and 28 with 20 mg whey, or treated with pWH-A, -B, -C or eWH homogenized in 0.5 mL PBS mixed with 10 μg cholera toxin (Quadratech Diagnostics, Epsom, UK) as an adjuvant. Non-sensitized mice received cholera toxin in PBS only (Fig. 1A). This resulted in 6 experimental groups: non sensitized mice (non-sens), whey-sensitized mice (whey-mice), mice treated with pWH-A, mice treated with pWH-B, mice treated with pWH-C and mice treated with an eWH (eWH-mice).

2.3.1. Allergic symptoms in response to whey challenge in murine model

On day 33, all mice were intradermally (i.d.) challenged with 10 μg whey (in PBS) in the right ear pinnae. As control, the left ear pinnae were challenged with PBS. After the i.d. challenge, the acute allergic skin response, body temperature and anaphylactic shock symptoms were determined 1 h after challenge. Anaphylactic symptoms were observed after intradermal challenge only. No changes in body temperature or shock score were observed upon oral challenge. To establish the severity of the shock, an anaphylactic scoring table (Table 1A and 1B) was used, as adapted from Li et al. (1999). To determine the acute allergen-specific skin response, ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). The allergen-specific net ear swelling was calculated by correcting the allergen-induced ear thickness for the basal ear thickness. Body temperature was measured rectally. On day 35, whey-sensitized mice and pWH- or eWH-treated mice received an oral challenge of 50 mg whey in 0.5 mL PBS. Thirty minutes after oral challenge, blood samples were

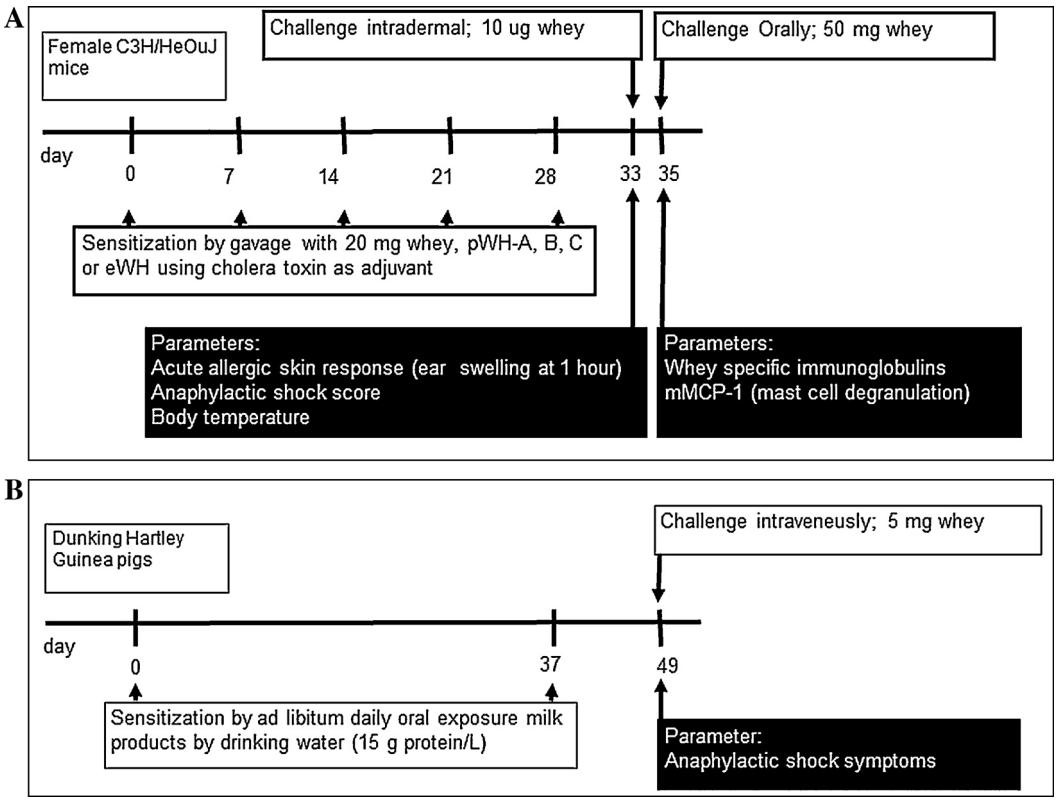

Fig. 1. Experimental set-up (A). Mice were sensitized weekly by i.g. gavage with **Whey**, or treated with pWH-**A, -B, -C** or extensively hydrolyzed whey protein (**eWH**) in PBS using cholera toxin (CT) as an adjuvant. Non-sensitized mice (non-sens) received CT in PBS only. On day 33 allergic skin responses, anaphylactic shock scores and body temperature were determined after intradermal Whey challenge in the ear pinnae. Whey-specific antibodies (IgE/IgG1) and mMCP-1 were measured on day 35, 30 min after i.g. Whey **challenge. (B) Guinea pigs** were sensitized by ad libitum daily exposure with **Whey**, or treated with pWH-**A, -B, -C** by drinking water for 37 days. After 12 days of rest Anaphylactic shock symptoms were determined after intravenous whey challenge on day 49.

Table 1A
Anaphylactic symptom scoring for mice.

Score	Symptoms
0	No symptoms
1	Scratching nose and mouth
2	Swelling around the eyes and mouth; pillar erecti; reduced activity; higher breathing rate
3	Shortness of breath; blue rash around the mouth and tail; higher breathing rate
4	No activity after stimulation, shivering and muscle contractions
5	Death

Table 1B
Anaphylactic symptom scoring for guinea pigs.

Score	Symptoms
0	No symptoms
1	Piloerection, nose licking or nose scratching and unrest
2	Above signs and/or tremors and sneezing or coughing
3	Above signs and/or urination, defecation, dyspnoea and ataxia
4	Above signs and/or convulsions
5	Above signs and/or death

collected. Blood samples were centrifuged at room temperature for 15 min at 13,500 rpm and sera were stored at −20 °C until further analysis for antibodies and mMCP-1.

2.3.2. Measurement of whey-specific antibodies and mMCP-1

Concentrations of whey-IgE and whey-IgG1 in serum were determined by means of ELISA as described previously (van Esch et al., 2010). In short, microlon plates (Greiner, Alphen aan de Rijn, the Netherlands) were coated with 20 µg of whey in coating buffer (Sigma Aldrich, Zwijndrecht, the Netherlands) for 18 h at 4 °C. Plates were washed and blocked for 1 h with buffer containing 50 mM Tris, 2 mM EDTA and 137 mM NaCl/0.05% Tween and 0.5% BSA. Serum samples were applied and incubated for 2 h at room temperature. Plates were washed and incubated with 1 µg/mL biotin labeled rat anti-mouse IgE/IgG1 (Pharmingen, Alphen a/d Rijn, the Netherlands) for one hour at room temperature. After washing, the plates were incubated with streptavidin-horse radish peroxidase (Sanquin, Amsterdam, the Netherlands) for one hour, washed and developed with *o*-phenylendiamine (Sigma Aldrich, Zwijndrecht, the Netherlands). The reaction was stopped after 10 min with 4 M H_2SO_4 and absorbance was measured at 490 nm on a Microplate reader. Results were expressed as arbitrary units (AU). Serum concentrations of mucosal mast cell protease-1 (mMCP-1) were determined according to the manufacturer's protocol using a commercially available ELISA kit (Moredun Scientific Ltd., Midlothian, UK).

2.3.3. Faecal DNA isolation, MITchip processing and analysis

Molecular fingerprinting of the composition of the colonizing microbiota was performed using MITChip analysis, a 16S rRNA-based phylogenetic array specifically designed to classify murine microbiota. To this end, fecal samples of the non-sensitized group (n = 7 or 8 per location) were collected at days -1, after acclimatizing, and 27. Samples were weighed and prepared for isolation using a standard bead-beating protocol (low scale bead-beating: 25 g 0.1 mm zirconia beads +3 glass beads in STAR buffer from Roche). DNA was extracted and purified with the Maxwell MDx (Promega), using the Maxwell 16 Tissue LEV Total RNA Purification Kit. The DNA was amplified using universal primers of the 16 s ribosomal RNA genes (Rajilic-Stojanovic et al., 2009), the FastStart Taq Polymerase and DNT Pack DNA amplification

kit (Roche Diagnostics). The used primers were T7prom-Bact-27-F (5′-TGA ATT GTA ATA CGA CTC ACT ATA GGG GTTTGATCCTGGCT CAG-3′) and Uni-1492-R(5′-CGG CTA CCT TGT TAC GAC-3′). DNA was purified with the High Pure PCR clean-up kit (Roche), according to the manufacturer's instruction, and the concentration was measured with NanoDrop-1000. Subsequently, transcription of DNA into RNA was performed using the RNAMAXX T7 Transcription Kit (Agilent Technologies). A part of the rUTP nucleotides was substituted with rUTPs labeled with an amino-allyl (0.5 μL rUTP and 1 μL amino-allylrUTP was added). The samples were purified with the RNeasy Mini-elute clean-up kit (Qiagen) and concentration was measured with NanoDrop-1000. Hereafter, RNA was labeled with CyDye: two post-labeling reactive dyes, Cy3 and Cy5 (GE Life Sciences), dissolved in DMSO. Each sample was labeled twice, once with each dye. Labeled RNA samples were purified with the Qiagen kit as described above. Subsequently, RNA was fragmented with the RNA fragmentation buffer from Ambion (60 min. 70 °C). The reaction was stopped with the accompanying stop solution (15 min., RT). Hybridization buffer (30 μL water, 7.5 μL 20x SSC and 1.5 μL 10% SDS) was added, and 40 μL of the mix was added onto the cover slide of the chip. The MITChip microarray was secured on top and incubated overnight (62.5° °C). The microarray was then washed in 50 mL 1x SSC, 0.3% SDS (room temp. 10 min.), in 50 mL 0.1x SSC, 0.3% SDS (45 °C, 10 min.), 50 mL 0.06x SSPE (room temp. 5 min.) and dipped in acetonitrile. Lastly, the microarrays were scanned with the high-resolution Agilent scanner (G2565CA). The MITChip scanning output data was analyzed with R scripts from https://github.com/microbiome and with the MySQL database (http://www.mysql.com) as described previously (Rajilic-Stojanovic et al., 2009; Geurts et al., 2011; Jalanka-Tuovinen et al., 2011; Lahti et al., 2011).

2.4. Experimental set-up guinea pig model

Sixteen weeks old male outbred albino, SPF-bred outbred Dunkin Hartley guinea pigs were raised and bred on a milk-protein free diet for at least two generations. Animals were fed a milk-free commercial guinea pig diet (viz. FD1(P)SQC, SDS Special Diets Services, Witham, England). The ambient temperature was maintained between 20 and 24 °C and relative humidity was maintained between 40% and 70% with a 12 h light/dark cycle. Animal care and use were performed in accordance with the general principles governing the use of animals in experiments of the European Communities (Directive 86/609/EEC) and Dutch Legislation (The Experiments on Animals Act, 1997). This included approval of the study by the ethical review committee (DEC number 2816). Guinea pigs were sensitized orally via the drinking water with whey, pWH-A, pWH-B, pWH-C which were administered at constant concentrations for 37 consecutive days (Fig. 1B). Guinea pigs are by nature more susceptible for the induction of allergic responses, therefore, no adjuvant is needed for allergic sensitization in guinea pigs. The energy-densities of the drinking water containing whey or pWH were equalized during sensitization by supplementation with maltodextrin (200 kcal/L). Non-sensitized guinea pigs received maltodextrine supplemented drinking water (200 kcal/L). After sensitization, all animals were kept on non-supplemented drinking water. The data on the allergenicity testing of pWH in the guinea pig were kindly provided by FrieslandCampina/TNO.

2.4.1. Anaphylactic shock symptom score to whey challenge in guinea pig model

On day 49, all guinea pigs were intravenously challenged in the penile vein with 5 mg whey in 0.5 mL PBS. After challenge, all animals were observed for 0.5 h and again at 3 h after challenge for signs of anaphylactic symptoms (Table 2, Fig. 1B).

2.5. Statistical analysis

Data was not normally distributed for whey-IgG1 and whey-IgE. whey-IgE data of center 3 was log transformed and ANOVA was used to analyze the data. Data was not normally distributed after log transformation for whey-IgE in centers 1 and 4 and in all centers for whey-IgG1. The Kruskal–Wallis test was used to analyze these data. mMCP-1 (all centers), body temperature (all centers) and acute allergic skin responses (center 1) were not normally distributed. Therefore, the non-parametric Kruskal–Wallis test was used to analyze the data, except for the acute allergic skin response in centers 3 and 4 were ANOVA with post-hoc Dunnet's analysis was used. These statistical tests were done with the graphpad Prism software (version 7.0). P-values are depicted as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, **** for $p < 0.0001$. Canoco5 was used to perform and visualize a Principal Component Analysis and a Redundancy Analysis on the MITChip output data. Pre-written R scripts (also available on Github) were used to create a dendogram for hierarchical clustering and diversity boxplots, and to perform tests such as Pearson correlations and Wilcoxon rank sum tests, along with a correction for multiple comparisons. SparCC, a python-based script was used to calculate similarities between species.

3. Results

3.1. Whey-specific antibodies

3.1.1. Whey-IgG1

All participating centers were able to immunize mice to whey as shown by elevated concentrations of whey-IgG1 (Fig. 2A). Notably, although in center 2 the whey-specific IgG1 levels were significantly increased in whey-mice, levels were remarkably high in non-sensitized mice, compared to the other centers, i.e. more than 100 times higher in AU. Significantly increased whey-IgG1 concentrations were observed in pWH-A mice in centers **1** and **4**, and in pWH-B- or -C mice in centers **1, 3** and **4** (Fig. 2A; Table 2). Treatment of mice with eWH did not result in increased whey-IgG1 responses and no whey-IgG1 responses were observed in center **2** for any of the modified whey-extracts.

3.1.2. Whey-IgE

In centers **1** (159 ± 38 vs 0), **3** (483 ± 199 vs 4 ± 2) and **4** (1416 ± 479 vs 2 ± 0) (Fig. 2B; Table 2) increased concentrations of whey-IgE were found in whey-mice, and in centers **1** and **3** in pWH-A mice (Fig. 2B, Table 2). pWH-B or pWH-C treated mice showed increased concentrations of whey-IgE in center **1**, **3** and **4**. Except for a low increase in whey-IgE in (23 ± 3) in center **4**, no elevated levels were observed in eWH-mice. In phase I of the validation process of the murine model (van Esch et al., 2013), a significant increase in whey-IgE and -IgG1 was set as a performance criterium. Since center **2** did not show a significant increase in whey-IgE analysis of allergic responsiveness data of that center was excluded (in Sections 3.2–3.4).

3.2. Acute allergic skin responses

As expected, positive acute allergic skin responses upon intradermal whey challenge were observed in whey-mice in centers **1, 3** and **4** (114 ± 23; 104 ± 10; 168 ± 9) compared to non-sens mice (36 ± 10; 21 ± 4; 33 ± 4, respectively) (Fig. 3A). Notably, no acute allergic skin responses were observed in pWH-A-treated mice (61 ± 13; 27 ± 6; 41 ± 10 respectively in centers **1, 3, 4**), although centers **1** and **3** showed elevated levels of whey-specific IgE concentrations in these mice. The skin response observed in pWH-B treated mice in center **4** (63 ± 6) was increased and the skin response in pWH-C treated mice showed an increase in center **1** (119 ± 18) which was similar to that in whey-sensitized mice. No positive skin responses were found in eWH-mice in any of the centers (Fig. 3A; Table 2). For this allergic parameter pWH-A is considered safe, pWH-B and -C are considered not safe

Table 2
Statistical overview of all data of phase II: Whey-IgE and whey-IgG1 for all centers. Based on IgE levels of center 2, the data of center 2 on all other allergic response parameters were excluded. All data are compared to non-sensitized control mice. (n.s.: statistically not significant).

Parameter compared to non-sensitized group	Mouse				Guinea pig
	Test group	Center 1	Center 2	Center 3	Center 4	
Whey-IgE	Whey	***	n.s.	****	****	
	A	***	n.s.	*	n.s.	
	B	*	n.s.	***	***	
	C	*	n.s.	**	***	
	eWH	n.s.	n.s.	n.s.	*	
Whey-IgG1	Whey	****	*	**	****	
	A	**	n.s.	n.s.	*	
	B	*	n.s.	*	***	
	C	****	n.s.	*	**	
	eWH	n.s.	n.s.	n.s.	n.s.	
Acute skin response	Whey	*		****	****	
	A	n.s.		n.s	n.s.	
	B	n.s.		n.s	*	
	C	*		n.s	n.s.	
	eWH	n.s.		n.s	n.s.	
mMCP-1	Whey	n.s.		***	*	
	A	n.s.		n.s.	n.s.	
	B	n.s.		n.s.	n.s.	
	C	n.s.		n.s.	n.s.	
	eWH	n.s.		n.s.	n.s.	
Temperature drop	Whey	**		n.s.	***	
	A	n.s.		n.s.	n.s	
	B	*		n.s.	n.s.	
	C	n.s.		n.s.	n.s.	
	eWH	n.s.		n.s.	n.s.	
Aphylactic shock score	Whey	6 out of 8		1 out of 8	7 out of 7	Yes (2 out of 2)
	A	No		No	No	No
	B	1 out of 6		No	No	4 out of 6
	C	2 out of 6		No	1 out of 6	3 out of 6
	eWH	2 out of 6		No.	No	Not measured
Number of positive Clinical parameters	*Whey*	*3/4*	–	*2/4*	*4/4*	
	A	*0/4*		*0/4*	*0/4*	
	B	*2/4*		*0/4*	*2/4*	
	C	*2/4*		*0/4*	*1/4*	
	eWH	*1/4*		0/4	0/4	

3.3. Mast cell activation (mMCP-1 release)

mMCP-1 serum concentrations were measured 30 min after oral whey challenge (Fig. 3B, Table 2). Elevated concentrations of mMCP-1 were observed in whey-mice versus non-sens mice in centers **3** (177 ± 47 vs 11 ± 3) and **4** (104 ± 50 vs 14 ± 3) indicating that mucosal mast cells were activated. In contrast to phase I (van Esch et al., 2013) no significant increases in mMCP-1 levels were observed in whey-mice in center **1** (93 ± 22 vs 39 ± 4) (Fig. 3B). Treatment of mice with pWH did not display a significant increase in mMCP1 compared to controls (Fig. 3B, Table 2). No elevated mMCP-1 levels were observed in eWH-mice. Based on this allergic parameter, pWH-A, pWH-B and -C can be considered safe (based on 2 centers).

3.4. Anaphylactic shock symptoms and body temperature

3.4.1. Mice

Whey-mice showed moderate to severe anaphylactic shock reactions after intradermal whey challenge in all three further evaluated centers (Fig. 4A, centers **1, 3** and **4**). In accordance to an absent acute allergic skin response in pWH-A treated mice we observed a zero anaphylactic shock score in pWH-A mice in centers **1, 3** and **4**. Minor anaphylactic shock symptoms were observed in pWH-B or -C treated mice in centers **1** and **4**, and except for center **1**, no anaphylactic shock score was determined in eWH-mice (Fig. 4A; Table 2). Anaphylactic shock symptoms in allergic mice were accompanied by a drop in body temperature. A profound decline in body temperature was observed in whey-mice in centers **1** (32 ± 1 vs 38 ± 0) and **4** (30 ± 1 vs 38 ± 0), but not in center **3** (37 ± 1 vs 38 ± 0 (Fig. 3C, Table 2). Body temperatures stayed around 38 °C in mice treated with pWH or eWH and was, except for pWH-B treated mice in center **1** not different from non-sens mice (Fig. 3C, Table 2). For this parameter pWH-A and pWH-C are considered safe (based on two centers).

3.4.2. Guinea pig

Two whey-sensitized guinea pigs developed anaphylactic shock score 5 and were considered positive. The challenge procedure of the remaining guinea pigs was cancelled for ethical reasons. In line with the acute allergic skin response and anaphylactic shock data as measured in the mouse food allergy model, no anaphylactic shock symptoms were observed in pWH-A treated guinea pigs. Anaphylactic shock symptoms were found in 4 out of 6 pWH-B treated guinea pigs and in 3 out of 6 pWH-C treated guinea pigs (Fig. 4B, Table 2). No anaphylactic shock data on eWH or allergen-specific IgE/IgG levels are available from the guinea pig model because eWH was not included in the guinea pig experiments which were performed separately and prior to the mouse

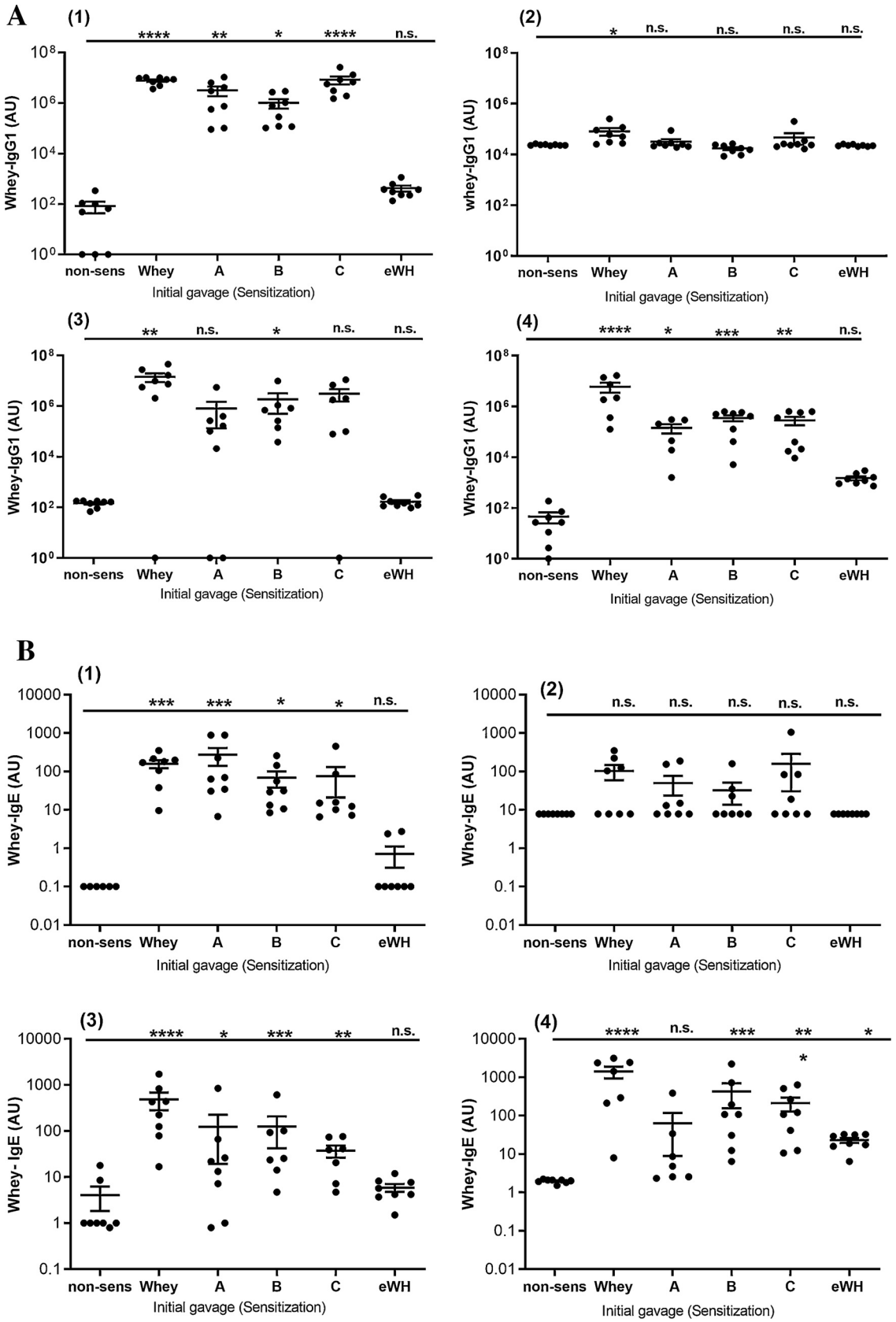

Fig. 2. (A) Allergen-specific IgG1. Concentrations of Whey-specific IgG1 were determined in sera of non-sensitized (non-sens) mice, Whey sensitized mice (**Whey**), pWH-**A, -B, -C** or **eWH** treated mice collected on day 35. (Data are expressed as individual values; *p < 0.05 **p < 0.01 ***p < 0.001 ****p < 0.0001 compared to non-sens mice). Numbers (1), (2), (3), and (4) indicate different centers.(B) Allergen-specific IgE. Concentrations of Whey-specific IgE were determined in sera of non-sensitized (non-sens) mice, Whey sensitized mice **(Whey)** or pWH-**A, -B, -C** or **eWH** treated mice collected on day 35. (Data are expressed as individual values; *p < 0.05 **p < 0.01 ***p < 0.001 ****p < 0.0001 compared to non-sens mice). Numbers (1), (2), (3), and (4) indicate different centers.

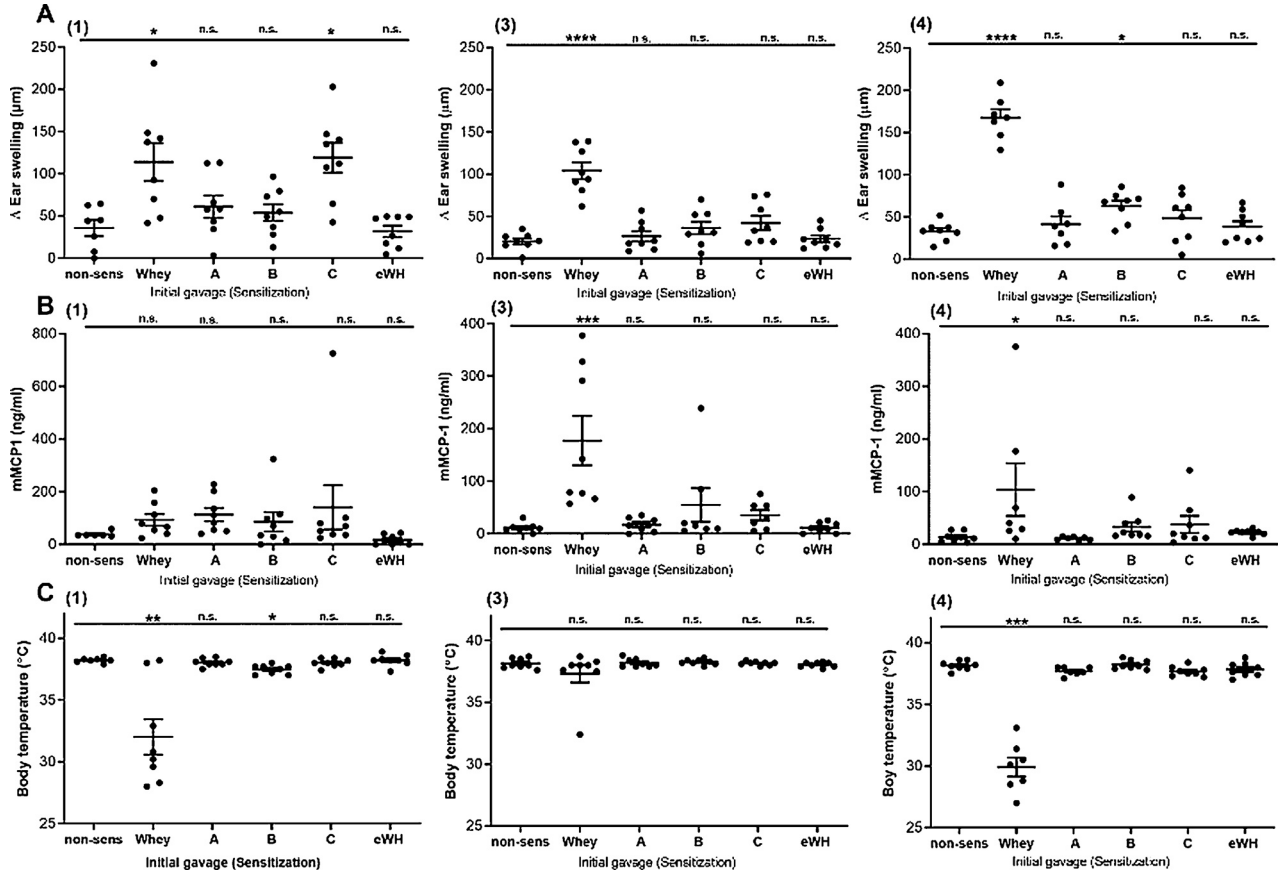

Fig. 3. Allergic symptoms in mice upon **Whey** challenge. (a) Acute allergic skin response, (b) mast cell activation and (b) drop in body temperature was determined after intradermal Whey challenge in non-sensitized (non-sens) mice, whey sensitized mice **(Whey),** pWH-**A, -B, -C** or **eWH** treated mice on day 33. (Data are expressed as individual values; $^{**}p < 0.01$ $^{**}p < 0.01$ $^{***}p < 0.001$ compared to non-sensitized mice). Numbers (1), (3), and (4) indicate different centers.

studies.

3.5. Intestinal microbiota composition in mice

Microbiota composition was analyzed in non-sens mice as a first step to get insight in the effect of housing on the robustness of the mouse model. To evaluate the overall differences between the samples from the four sites tested, beta diversity was calculated as a measure of the microbiota variance. Principal Component Analysis (PCA) indicates clear separation between samples and center **4** can be distinguished from the other groups based on microbiota composition, already a few days after arrival on the test location (Fig. 5 and Supplementary Fig. 1). For clarity the centroid of each site is represented (Supplementary Fig. 1 squares). The microbial community composition varies by individual and by site the position of the centroids indicate the degree of variation between sites.. Within each center no major changes were observed throughout the study (from day -1 to day 27) indicating that the housing of the animals was the main factor for the observed clustering of center 4 and no major effect due to the treatment (Fig. 5). When focusing on contribution of bacteria taxa there is higher abundance of Unclassified Porphyromonadaceae in samples of center **4** (Supplementary Fig. 1).

4. Discussion

In the current multicenter ring trial, the discriminatory power and the sensitivity of the mouse allergy model to predict the residual allergenicity of hydrolyzed whey-proteins was investigated and compared to the frequently used guinea pig model. Overall, three of the 4 centers involved in the trial showed comparable patterns with regard to the various immunological and clinically relevant allergic parameters that were analyzed in the mouse model. Particularly, results showed that responses to whey, pWH were comparable between the three centers and, as far as tested, in line with the allergic shock score in the guinea pig model. In one center (center 2), levels of whey-specific IgE were not increased despite the increased levels of specific IgG1. Previously, we previously considered the increased IgE levels an performance criterium and therefore did not further evaluate the data of the other parameters obtained in this center. In all three other research centers, whey-mice showed elevated levels of whey-IgE and mostly clear allergic symptoms upon challenge with whey.

The pWH preparations elicited whey-specific IgE responses in almost all cases, whereas allergic responses were generally absent or very modest (e.g. temperature drop in center 1 for pWH-B, ear swelling in centers 1 and 4 for pWH-C and -B, respectively). In comparison, the guinea pigs showed anaphylactic responses to pWH-B and -C, but not to pWH-A. So, despite the presence of whey-IgE in most centers, pWH-A-treated mice showed no significant increase in clinical parameters (summarized in Table 2). The molecular weight distribution of the three hydrolyzed proteins is comparable and offers no explanation for the differences in allergenicity between pWH-A and pWH-B/C, indicating the importance of assessing allergic responses for safety testing. In all, our current data in mice are in line with guinea pig-data showing complete absence of anaphylactic shock symptoms only in pWH-A. In addition, the mouse and guinea pig data were also comparable, and provided similar conclusions on sensitizing potential of the different pWH preparations. Based on these pre-clinical animal data the pWH-A is considered safe for market introduction. The hydrolyzed proteins as used in this ring-trial are not available on the market so there are no postmarketing data available.

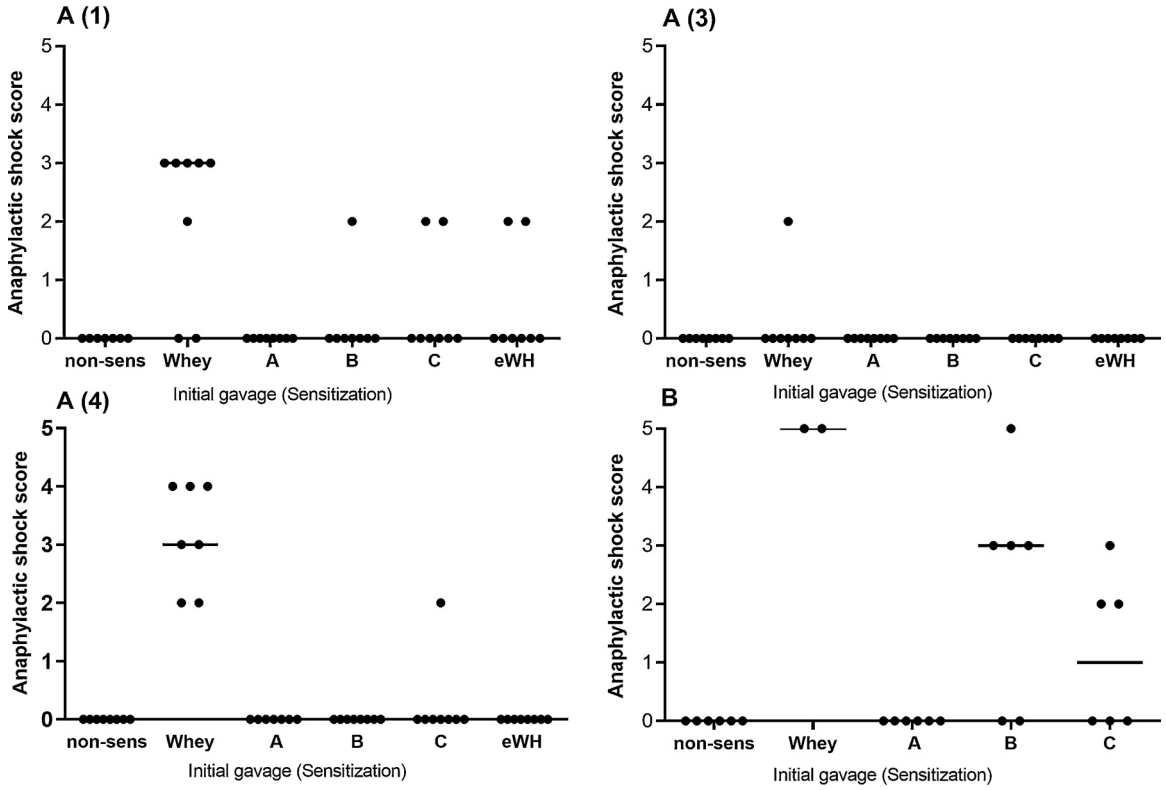

Fig. 4. Anaphylactic shock symptom scores in mice and guinea pig upon whey challenge. (A) Anaphylactic shock scores were determined after intradermal Whey challenge in non-sensitized (non-sens) mice, Whey sensitized mice **(Whey),** pWH-**A, -B, -C** or **eWH** treated mice on day 33. (Data are expressed as individual values). Numbers (1), (3), and (4) indicate different centers. (B) Anaphylactic shock scores were determined after intravenous Whey challenge in non-sensitized (non-sens), whey sensitized **(whey)** or pWH-**A, -B, -C** treated guinea pigs on day 49. (Data are expressed as individual values).

Noted disadvantages of using guinea pigs, however, are that they may experience severe discomfort as we also found in this study in the positive control group, and in addition, that sensitization is based on only one clinically related parameters measured as an anaphylactic shock response and (anaphylactic) antibodies of IgG1a isotype (Zosky and Sly, 2007). Since the latter is not IgE, which is the main anaphylactic isotype in allergic humans, extrapolation of guinea pig data to man is difficult. Importantly, the mouse food allergy model is IgE-mediated and in addition allows detection of various additional clinical parameters in the same mice (Abbring et al., 2017; Vonk et al., 2017a; van den Elsen et al., 2014; van Esch et al., 2013).

The latter is of importance because current murine data shows that positive IgE levels alone are not sufficient to predict an allergic response of hydrolyzed proteins. It is indeed also known for humans that allergen-specific IgE does not always induce allergic clinical symptoms (Anvari et al., 2019), and that, although allergen-specific IgE is considered an important parameter reflecting sensitization to the allergens, food challenges are needed to confirm allergic responses. None of the centers was able to differentiate between the sensitizing capacities of the pWH (B and C)-treated mice based on a single allergic parameter, stressing that indeed measurement of multiple parameters is important to give a proper prediction of the residual allergenicity and safety of hydrolyzed infant formulas for human use. Thus, since the mouse model provides the same conclusion as the guinea pig model with regard to sensitizing potential, the mouse model is preferable above the guinea pig model. For now it is difficult to judge which parameter predicts the residual allergenicity of hypoallergenic infant formulas best in humans since the pWH tested in this ring-trial are not available on the market so there are no post marketing data available.

An important area of concern is the high variability of allergic read-outs after challenge in whey sensitized and challenged animals. In center 3, whey-mice did not show an anaphylactic response (based on temperature drop and clinical score) and mMCP1-responses in whey-mice did not deviate from controls in center 1. Previously however, murine parameters varied less in our previous first phase study of this multi-center ring trial (van Esch et al., 2013). It should be noted that we assured that mice, which were from the same strain, gender and breeding batch, received the same allergen-free feed (over more than 2 generations), and that experimental set-up and methodological protocols were standardized. But this apparently does not prevent the variability and in addition did not prevent that the sensitization (induction of IgE) failed in center 2. Because it is known that the microbiome plays a crucial role in the immunological homeostasis of the individual (Bjorksten et al., 2001; Thompson-Chagoyan et al., 2010), we performed a first fecal microbiota analysis in fecal samples of non-sensitized mice. The fecal microbiota showed no significant changes in alfa-diversity (richness) for any of the timepoints or centers (data not shown). Interestingly, samples collected at research center 4 clustered separately from the others, already a few days after arrival on the test location. No further changes were observed throughout the study (from day −1 to day 27) indicating that adapting to local facilities after the transport of the animals from breeder to the different centers was the main factor for the observed clustering of center 4. This clustering of center 4 is mainly driven by a higher abundance of 'Unclassified Porphyromonadaceae' (Supplementary figure). We can only speculate that a changed abundance of gut microbiota before sensitization underlies the differences between center 4, where all allergic read-outs showed positive in whey-sensitized and -challenged mice (positive control) and centers 1 and 3, where not all allergic symptoms showed positive in whey sensitized and challenged mice. The microbial composition of center 2 was comparable to center 1 and 3, providing no explanation for the lack of significant levels in IgE as observed in center 2. Future, more specific studies are needed to further investigate the relation between differences in gut microbiota and the allergenicity of whey and other

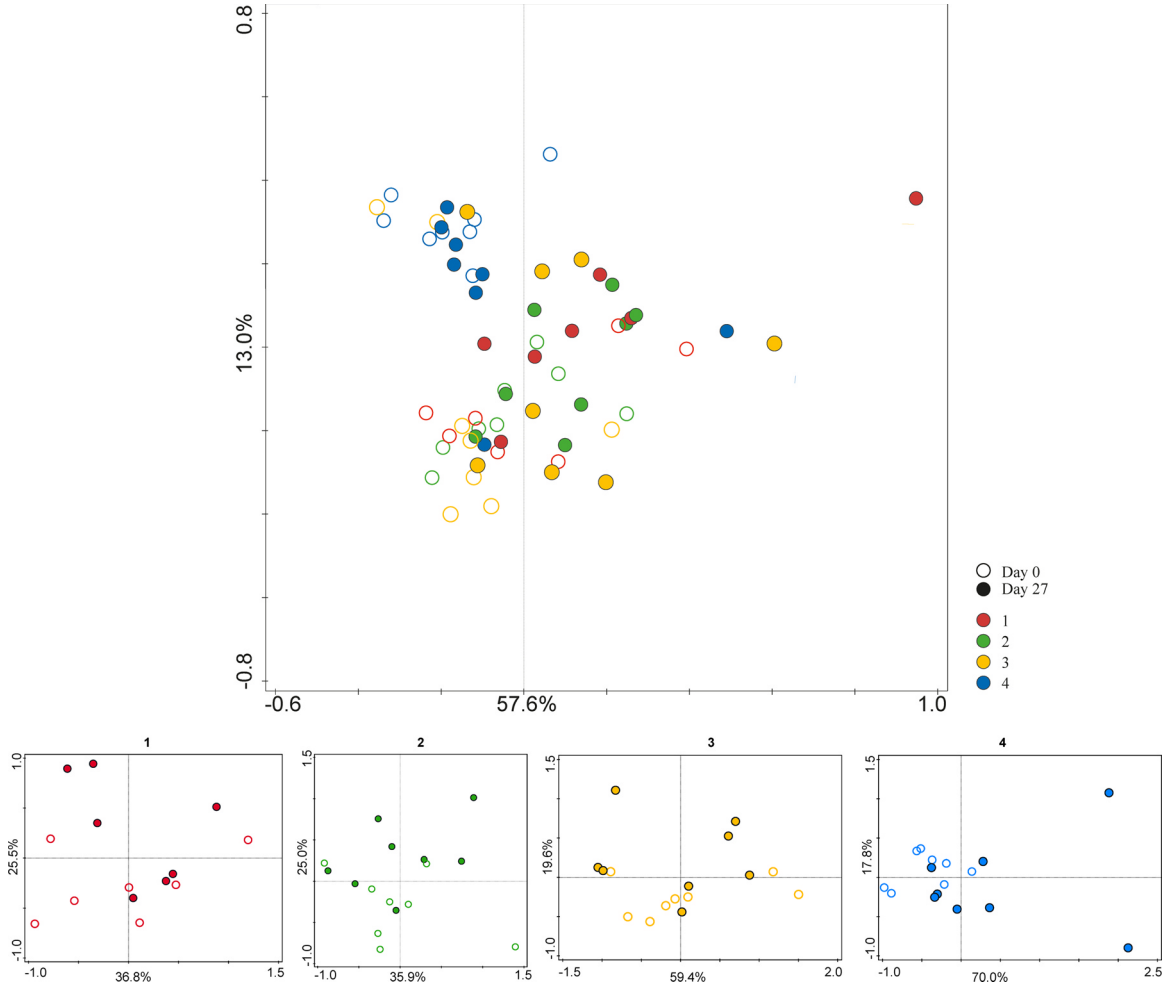

Fig. 5. Principal component analysis of fecal samples distinguished center 4 from other centers. Principal Component analysis derived fecal samples of non-sensitized mice. The samples of the four locations are plotted in one figure (top), each location is also plotted separately (bottom). The PCA analysis of microbiota in fecal samples shows that research center 4 cluster separately from the others. Each center shows minimal effect over time.

food proteins.

So, concluding from the two phases of this multi-center trial it is clear that the mouse food allergy model is a good candidate to replace the Guinea pig assay. The measurement of multiple parameters is important to give a proper prediction of the residual allergenicity and safety of hydrolyzed infant formulas for human use. Thus, since the mouse model provides the same conclusion as the guinea pig model with regard to sensitizing potential, the mouse model is preferable above the guinea pig model. The high variability may be an issue of concern but may also be inherent to the complexity of processes and external influences (microbiome, housing) involved in sensitization and allergic responses. The main future challenge will be to develop an overall scoring system for proper risk assessment, taking all parameters into account. For this, data on more preparations, including of those that are available on the market are needed.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxlet.2020.05.020.

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