The Journal of Allergy and Clinical Immunology: In Practice

Continuous rather than solely early farm exposure protect from hay fever development

--Manuscript Draft--

Manuscript Number:	INPRACTICE-D-22-00656R1
Article Type:	Original Article
Keywords:	Childhood, farm milk, farming, gut microbiome, hay fever, animal sheds.
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Abstract:	Background: An important ‘window of opportunity’ for early life exposures has been proposed for the development of atopic eczema and asthma. Objective: However it is, unknown whether hay fever with a peak incidence around late school age to adolescence is similarly determined very early in life. Methods: In the PASTURE birth cohort potentially relevant exposures such as farm milk consumption and exposure to animal sheds were assessed at multiple time points from infancy to age 10.5 years and classified by repeated measure latent class analyses (N=769). Fecal samples at age 2 and 12 months were sequenced by 16S rRNA. Hay fever was defined by parental reported symptoms and/or physician’s diagnosis of hay fever in the last 12 months using questionnaires at age 10.5 years. Results: Farm children had half the risk of hay fever at age 10.5 years (adjusted odds-ratio (aOR) [95% CI]=0.50 [0.31; 0.79]) compared to non-farm children. While early life events such as gut microbiome richness at age 12 months (aOR=0.66 [0.46; 0.96]) and exposure to animal sheds in the first three years of life (aOR=0.26 [0.06; 1.15]) were determinants of hay fever, the continuous consumption of farm milk from infancy up-to school age was necessary to exert the protective effect (aOR=0.35 [0.17; 0.72]).

	Conclusion: While early life events determine the risk of subsequent hay fever, continuous exposure is necessary to achieve protection. These findings argue against the notion that only early life exposures set long-lasting trajectories.
Additional Information:	
Question	**Response**
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	authors have no conflict of interest in relation to this work. J.G: reports payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from The journal Pediatric Allergy and Immunology (co-owned by Wiley and EAACI); personal honorarium for serving as Associate Editor. M.K: reports grants or contracts from European Union, German Ministry of Education and Research, German Research Foundation, Infectopharm; consulting fees from Bionorica, Sanofi, Novartis, Bencard; payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from ERS, EAACI, ATS, Novartis, Glaxo, Chiesi, Sanofi, Nutricia, Hipp, Allergopharma; patents planned, issued or pending METHOD FOR TESTING A SUBJECT THOUGHT TO HAVE OR TO BE PREDISPOSED TO ASTHMA European patent application 5 EP07301135.5. Other co-authors declares no conflict of interests.
Please enter the text of the Highlights Box, copied from your manuscript document. Each Original Article MUST be accompanied by a highlights box, placed in the text after the Abstract, that provides bulleted answers to the following questions (each answer should be no longer than 35 words): 1. What is already known about this topic? 2. What does this article add to our knowledge? 3. How does this study impact current management guidelines?	1. What is already known about this topic? The protective effects of early life farm exposures and gut microbiome composition on atopic diseases and asthma proposes an important window of opportunity. 2. What does this article add to our knowledge? Early life farm exposures also determine risk of hay fever. However, continuous farm milk consumption is necessary for optimal prevention, thereby arguing against the notion of an early-determined trajectory governing later outcomes. 3. How does this study impact current management guidelines? These results emphasize the preventive potential of continuously drinking unprocessed farm milk for hay fever protection, suggesting carrying out clinical trials to test microbiologically safe cow’s milk for protection from hay fever.

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11. Oktober 2022

Subject: Submission of the revised manuscript as an “Original article” to The Journal of Allergy and Clinical Immunology: In Practice

Dear Prof. Dr. Schatz,

Please find enclosed the revised manuscript entitled ‘Continuous rather than solely early farm exposure protect from hay fever development’ with manuscript number “INPRACTICE-D-22-00656” along with point-to-point response to the reviewers and editorial office to be considered for publication as an original article in The Journal of Allergy and Clinical Immunology: In Practice.

The content of this paper has not been published, nor is it under consideration for publication elsewhere. All the authors have read the revised manuscript, and approved its submission for publication.

We are of the opinion that the present findings will be of interest to the readers of The Journal of Allergy and Clinical Immunology: In Practice, and thank you for considering our work for publication. We look forward to your response.

Sincerely yours,

Sonali Pechlivanis

Response to the reviewers

Dear Editor,

We thank you, the reviewers and the Editorial Office for the constructive comments that has helped us improve our manuscript. We would like to resubmit the attached revised version of the manuscript to be considered for publication in the JACI: In Practice journal.

Please find our point-to-point response to the questions raised by the reviewers and the editorial office:

COMMENTS FROM REVIEWER #1:

This multi-center birth cohort study examined the association between early farm exposure and hay fever development at the age of 10.5 years. It reported that early life exposure to farm environment could protect against subsequent hay fever, however, the optimal prevention was seen in continuous exposure. This study highlights the significance of continuous exposure to farm environment, rather than only early life exposure in the prevention of hay fever. Following please see some comments:

1. **The major concern is the definition of outcome. Because this is a prospective birth cohort, time, or age of hay fever diagnosis and/or symptoms should be available as it stated that some of the children had reported hay fever symptoms or diagnosis at the age 4 or 5 (line 401-403). Incidence of hay fever (onset of the disease) should be analyzed or examined.**

We thank the reviewer for pointing this out. Since it is a prospective birth cohort, we have now modified the discussion section lines 446-448 as follows: “In fact, 4.6%, 5.9% and 6.7% of children with data on hay fever at age 10.5 years had already reported symptoms and/or a diagnosis of hay fever at age 4, 5 and 6 years, respectively.”.

At 10.5 years, incidence of hay fever was 7.7% (N=48). In our manuscript, we have already reported the incidence of hay fever at age 10.5 years. The following is written in the results section lines 369-373 “The inverse association of ‘continuous consumption of farm milk’ compared to ‘no consumption of farm milk’ was still observed when using the stringent definition of hay fever (0.41 [0.17; 0.97], *0.04*) (Figure E2 Online Repository Text) or incident hay fever at age 10.5 years (0.39 [0.15; 0.99], *0.05,* data not shown).”. And in the methods section (Online Repository Text) lines 73-77 “Incident hay fever at 10.5 years (N=48) was defined by parent reported symptoms (itchy, runny, or blocked nose without a cold accompanied by red itchy eyes) and/or a physician’s diagnosis of hay fever in the last 12 months using questionnaires at age 10.5 years and excluding those having hay fever before the age of 10.5 years.”.

2. Among the participants, how many of them have developed other allergic disease, such asthma, eczema, or food allergy? Most importantly, how many children in the no hay fever (controls) have other allergic disease?

The children without hay fever when compared to with hay fever had lower prevalence of asthma (6.2% vs 28.9%), eczema (9.6% vs 36.7%), and food allergy (3.1% vs 21.7%) (Table 1 in the revised manuscript).

We have now added the following regarding other allergic diseases in the Table 1 and in the results section lines 344-346 “Hay fever at the age of 10.5 years was reported in 12.9% children. Of these, 28.9%, 36.7%, and 21.7% had asthma, eczema, and food allergy at age 10.5 years respectively (Table 1).”

3. Because the exposure of interests are repetitive measurements, longitudinal LCA, or repeated measures LCA (RMLCA), an extension of LCA should be applied. Please clarify that RMLCA was used in the analysis.

Thank you for pointing this out. Since the exposures are measured at several time points, we have used repeated measure LCA. We have added the following in the methods section line 293 “We performed repeated measure latent class analyses (RMLCA)” and replaced acronym LCA with RMLCA everywhere in the manuscript.

4. How was the farm and non-farm groups defined? And what was the reason that 193 children did not have hay fever status?

We have now added the following in the methods section lines 253-256 “Children of mothers living on family-run livestock farms at birth of the children were assigned to the farm group. The non-farm group included children of mothers from the same rural areas but not living on a farm (18).”.

Thank you for this hint. Sorry for the confusion regarding 193 children. These are the children who had data on farm exposures i.e. consumption of farm milk and exposure to

animal sheds from pregnancy till 10.5 years (N=962) at least at one time point and hay fever (N=769) at 10.5 years.

At baseline, 1133 children were enrolled into the PASTURE birth cohort. Of them, 778 children participated in the 10.5 years examination and 769 children had data available on hay fever and were included in the present study. Hence, 364 of the original cohort and not 193 children were not included. In the revised version, we have now corrected and modified the results section lines 336-340 and the Table E1 (Online Repository Text) as follows “At 10.5 year follow up 778 children participated in the PASTURE study and 769 have data on hay fever. Comparing the baseline characteristics between included (N=769) and excluded children (N=364) did not show any significant difference except for maternal age at pregnancy, maternal smoking, parental education, and premature birth (Table E1 Online Repository Text).”.

5. It is unclear how mediation analysis of gut microbiome in the association between fam milk consumption and hay fever was conducted. Please specify the analytical method that was used.

We have now added the following in the methods section lines 329-330 “The mediation analysis was conducted through path analysis using maximum likelihood test to estimate the regression parameters in Mplus 8.5 (22).”

6. It was unclear what covariates were included in the multivariate analyses. It is also rather difficult to follow the results (too many figures and tables). It would be helpful to consolidate and reorganize the results to limit the numbers of figures and tables.

The models were adjusted for centers, growing up on a farm and parental asthma and/or atopy. The following is already mentioned in the methods section lines 320-323 "The above models were adjusted for centers and confounders (growing up on a farm and parental asthma and/or atopy) associated with hay fever and exposures in our study. No other confounders i.e. associated with both outcome and exposures were found.".

As suggested, we have now excluded Figure E5 and Tables (E4, and E5) from the "Online Repository Text" and included the respective OR [95%CI] in the text in the results section lines 377, 382, and 408.

7. In Table E4, the analysis stratified by study centers could not provide valid results due to small numbers in each category. Similarly, the sample size was too small to be interpreted appropriately in the analysis that excluded children having a family history of parental atopy and avoided milk or milk product.

We are aware that the numbers are small. Nevertheless, the effects are comparable in 'continuous consumption of farm milk' compared to 'no consumption of farm milk' especially in the analysis that excluded the children having a family history of parental

atopy and avoided milk or milk product. As suggested, we have now deleted Table E4 from the “Online Repository Text” and included the text in the results section line 377.

8. The assessment of milk consumption was based on the frequency (daily, 1-6 times a week, less than once a week, or no consumption). Was the volume or size of the milk consumption measured? If not, it was misleading to use "amount" of milk consumption. The "frequency" of milk consumption should be used.

We thank the reviewer for pointing this out. The assessment of milk consumption was based on frequency and not on volume or size of milk consumed. We have now replaced “amount” with “frequency”. We do have information on the volume of farm milk consumed. However, this information was not available at three time points (age 12, 18 and 24 months). Hence, the assessment of milk consumption was based on frequency rather than volume of farm milk consumed.

9. Maternal/prenatal exposure to smoking, medication use, maternal age has been associated with allergic disease and hay fever. Was this information available in the PASTURE cohort? It should be considered or addressed.

10. Several other important confounders were not considered, including indicator of socioeconomic status (i.e. household income, parental education, access to health care), second-hand smoking, use of antibiotics, prematurity, birth weight or body mass index of the children.

Thank you for this information. As suggested, we have now addressed these variables (maternal age at pregnancy, maternal smoking, second hand smoking, parental education, use of antibiotic during pregnancy, premature birth, birth weight and use of antibiotics during first year of life; Table 1 and Table E1) in the revised manuscript. However, none of these exposures showed any statistical significant association with hay fever in our study.

COMMENTS FROM REVIEWER #2:

Very nice analysis and manuscript. Only minor comments.

1. Is there a way to calculate a "number needed to treat" re: farm milk's protective effect against hay fever? for example, how many children should drink continuous consumption of farm milk to prevent a diagnosis of hay fever?

As suggested by the reviewer, we have now calculated the Number Needed to Treat using an R-script available at https://rpubs.com/RatherBit. However, this was not a randomized placebo-controlled double-blind trail and thus numbers must be taken with some caution. In the revised version, we have added the following in the methods section lines 323-325 “We additionally calculated the Number Needed to Treat (NNT), which is the effectiveness of a treatment on an outcome using an R-script (22).”, in the results section lines 394-396 “Additionally, NNT calculated in our study was 7.14, i.e. 7 children would have to drink farm milk continuously from pregnancy by mothers until age 10.5 years in order to prevent hay fever in one child.”, and in the discussion section lines 497-499 “Further, the NNT in our study was 7, however, this study is not a randomized

placebo-controlled double-blind trail and thus numbers must be taken with some caution.”.

2. thinking about benefits and risks of farm milk, is there any information to consider any adverse events from drinking farm milk continuously -- for example, were data collected on gastrointestinal illnesses or other possible side effects of farm milk?

In PASTURE study “gastrointestinal illnesses” such as diarrhea was monitored. In the previous study by Loss et al. (1) no clear association of milk consumption with diarrhea in infancy was observed. Similarly, at 10.5 year no elevated risk of diarrhea and farm milk consumption was observed (Table 1 below). We also looked at BMI and did not find any association of farm milk consumption and BMI. Also, the study by Karadag et al. (2) showed no relation of farm milk consumption to eczema or rash.

Table 1.

	Farm milk (Yes) N=290	Farm milk (No) N=474	*P-value*
Diarrhea Yes (N=379) No (N=385)	 135 (46.6) 155 (53.5)	 244 (51.5) 230 (48.5)	 *0.21*
BMI (kg/m^2)*	17.9±2.8	17.6±3.1	*0.16*

* mean±SD

We have added the following in the revised version of manuscript in the discussion section lines 490-491 “An elevated risk of diarrhea and farm milk consumption at 10.5 years was not observed (data not shown).”, lines 494-497 “Hence, the Milk Against Respiratory Tract Infections and Asthma (MARTHA) an ongoing interventional trial is being carried out to evaluate the preventive effect of minimally treated, i.e. only pasteurized and thus microbiologically safe cow’s milk on upper respiratory tract infections and allergy (34).” and on lines 522-527 “These results emphasize the preventive potential of continuously drinking unprocessed farm milk for hay fever protection. However, the risks associated with raw cow’s milk consumption prohibit its recommendation for daily life. The results of the MARTHA trial however will shed light on potential side effects (34). Further clinical trials based on the present results are warranted.”

3. would you consider adding or highlighting potential next steps or future studies that could build on the current findings?

We have now added the following in the discussion section lines 522-527 “These results emphasize the preventive potential of continuously drinking unprocessed farm milk for hay fever protection. However, the risks associated with raw cow’s milk consumption prohibit its recommendation for daily life. The results of the MARTHA trial however will shed light on potential side effects (34). Further clinical trials based on the present results are warranted.”

4. lines 199-200: current wording implies quality of life (QoL) is a "socio-economic factor", which might be debatable -- could consider deleting "socio-economic factor" to be more clear. Also for this text, to increase generalizability, could consider adding references from outside of Europe, e.g., https://pubmed.ncbi.nlm.nih.gov/33754932/

We thank the reviewer for the suggestion. We have now deleted the word “socio-economic factor” and added the suggested literature by Tkacz et al.

5. line 420: consider removing the apostrophe from "it's".

As suggested, we have now removed apostrophe from “it’s” (line 465).

COMMENTS FROM REVIEWER #3:

The authors are to be complimented on this very well written and clearly reported study, analyzing the PASTURE cohort. The results are supportive of the study conclusion. I have only two minor suggestions:

1. Study could be strengthened by providing sample size justification,

We thank the reviewer for the suggestion. In our revised version, we have now added the posthoc power calculation in the discussion section lines 508-513, “We performed a posthoc power calculation using SAS and considering α=0.05 (two-sided). For our sample size of 650, i.e. in the exposure groups ‘continuous consumption of farm milk’ and ‘no consumption of farm milk’ the power of study is over 80% assuming the response

probabilities ranging from 0.02-0.18 for having hay fever in children who consume farm milk and unadjusted OR of 0.24. Thus, our study was well powered to detect a relatively strong effect of farm milk consumption on hay fever."

2. The study might wish to acknowledge potential caveats in observational study and consider taking more modern causal inference approach in the future.

Yes, the reviewer is right. It is an observational study which has its potential caveats and hence we refer to the MARTHA trial which is an ongoing intervention trial with minimally processed, i.e. only pasteurized cow's milk. We have added the following in the discussion section lines 491-497 "The results of the present study show protective association of continuous consumption farm milk on hay fever. However, one of the potential caveats of the observation study is finding causality. Hence, the Milk Against Respiratory Tract Infections and Asthma (MARTHA) an ongoing interventional trial is being carried out to evaluate the preventive effect of minimally treated, i.e. only pasteurized and thus microbiologically safe cow's milk on upper respiratory tract infections and allergy (34)."

COMMENTS FROM THE EDITORIAL OFFICE:

**** Please note that any groups listed as authors must have a conflict of interest statement listed for each group member. Otherwise, please change the author listing to "on behalf of X group."**

We thank the editorial office for pointing this out. We have now obtained the conflict of interest statement from the members of the PASTURE study group and moved the list of PASTURE study members from the Acknowledgement section to the list of author's lines 39-54. However, I still have a question regarding PASTURE Study group members. Will the PASTURE Study group members be listed in Pubmed? If not, please suggest the possibility of listed them in Pubmed.

**** Revise the abstract to comply with the Journal's structured format, which has five headings: Background, Objective, Methods, Results, Conclusion. When revising your abstract, please keep in mind our length guidelines, which are 250 words or less.**

As suggested, we have now included the five headings in the abstract with the word count of 239.

**** Please provide a Highlight Box that provides bulleted answers (no more than 35 words each) to the following questions:**

We have added the following on lines 207-217

1. What is already known about this topic?

The protective effects of early life farm exposures and gut microbiome composition on atopic diseases and asthma proposes an important window of opportunity.

2. What does this article add to our knowledge?

Early life farm exposures also determine risk of hay fever. However, continuous farm milk consumption is necessary for optimal prevention, thereby arguing against the notion of an early-determined trajectory governing later outcomes.

3. How does this study impact current management guidelines?

These results emphasize the preventive potential of continuously drinking unprocessed farm milk for hay fever protection, suggesting carrying out clinical trials to test microbiologically safe cow's milk for protection from hay fever.

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As suggested, we have used numbering function and numbered each line of the manuscript and the “Online Repository Text”.

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As suggested, eFigure’s are now uploaded separately.

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We have now renamed the supplemental methods as “Online Repository Text”

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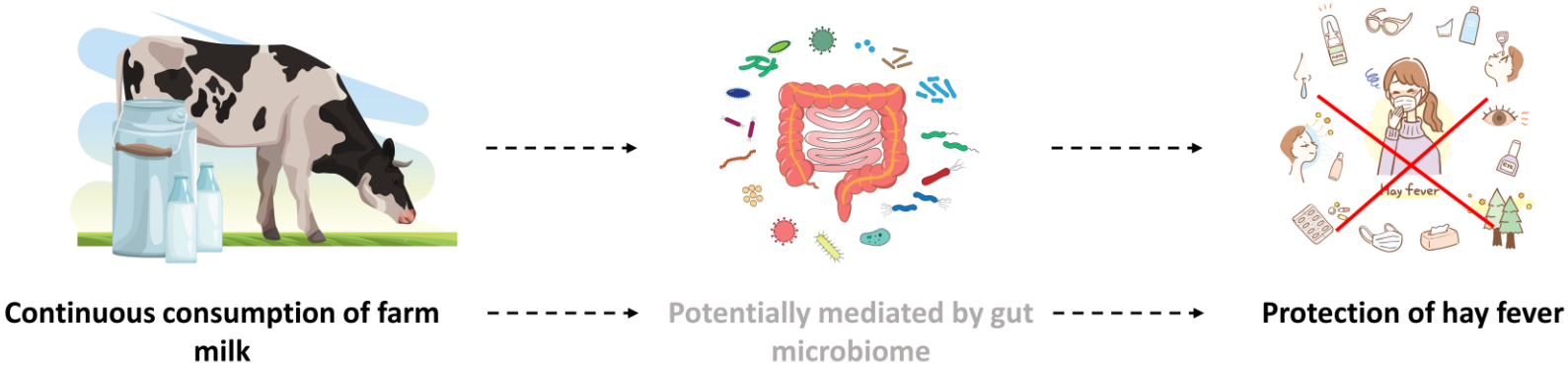

1. Loss G, Depner M, Ulfman LH, van Neerven RJ, Hose AJ, Genuneit J, et al. Consumption of unprocessed cow's milk protects infants from common respiratory infections. J Allergy Clin Immunol. 2015;135(1):56-62.
2. Karadag B, Ege MJ, Scheynius A, Waser M, Schram-Bijkerk D, van Hage M, et al. Environmental determinants of atopic eczema phenotypes in relation to asthma and atopic sensitization. Allergy. 2007;62(12):1387-93.

Continuous rather than solely early farm exposure protect from hay fever development.

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Source of funding: The PASTURE study was supported by the European Commission (research grants QLK4-CT-2001-00250, FOOD-CT-2006-31708 and KBBE-2007-2-2-06), the European Research Council (grant 250268), Deutsche Forschungsgemeinschaft, German Center for Lung Research (DZL), Kühne Foundation, French national programme for hospital research, EVO- and VTR-funding, the Academy of Finland (grant 139021, 287675, 338679, 339666), the Yrjö Jahnsson foundation, and EU, Academy of Finland and smaller grants from Finnish foundations supporting medical research (Juho Vainio Foundation, Päivikki and Sakari Sohlberg foundation, Finnish cultural foundation). The current analyses were supported by the Bavarian State Ministry for Health and Care.

Conflict of interest:

S.P.: reports support for attending virtual ERS International Congress 2021. C.R.: reports payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from ALK, University of Bern. C.S.: reports grants from Universities Giessen and Marburg Lung Center, German Center for Lung Research, University Hospital Giessen and Marburg research funding, Deutsche Forschungsgemeinschaft (DFG) (SFB1021 (C04), KFO 309 (P10), SK 317/1-1 (Project Nr. 428518790)), TransMIT, Mead Johnson Nutrition; Consulting fees: Hycor Biomedical, Bencard Allergie, Thermo Fisher Scientific; Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events: Hycor Speaker Bureau; Support for attending meetings and/or travel: Hycor, Bencard Allergy; Participation on a Data Safety Monitoring Board or Advisory Board: Hycor

Biomedical, Bencard Allergy; Leadership or fiduciary role: ESGREV, DZL ALLIANCE; Receipt of equipment, materials, drugs, medical writing, gifts or other services: Bencard Allergie. C.B.: reports support for attending meetings/travel from GSK, Sanofi, Chiesi, Novartis, AstraZeneca. M.J.E.: reports support for the present manuscript from German Center for Lung Research (DZL); Grants or contracts from any entity: Dutch Longfonds; Patents planned, issued or pending: EP000001964570B1, US000009950017B2, EP000002361632B1; Receipt of materials: Milk powder from Friesland Campina. A.D-C.: reports support for the present manuscript from French national programme for hospital research; Grants/contracts: French public agency ANSES, Don du Souffle for the PASTURE 16-yr Visit, Fondation du Souffle for the PASTURE 16-yr visit, Novartis, ARAIRLOR (Association Régionale d'Aide aux Insuffisants Respiratoires de Lorraine); Consultant fees: Stallergens, ALK, Aimmune Therapeutics; Payment/Honoria: Aimmune Therapeutics (speaker at symposium at the EAACI congress in 2019 and at the french congress of Allergy in 2019), Novartis; Attending meetings: Mead Johnson for Pediatric Allergy and Asthma Meeting 2019, Nutricia for EAACI congress 2019, Aimmune Therapeutics for EAACI congress 2019, Novartis for the French congress of Allergy 2019; Stock: Essilor Luxottica. B.S. reports support for the present manuscript from DFG, and EU; Grants/contracts: DFG, and EU. R.L.: reports support for the present manuscript from Kühne Foundation, and EU European Commission (research grant QLK4-CT-2001-00250); A.M.K: reports support for the present manuscript from EVO- and VTR-funding, the Academy of Finland (grant 139021, 287675, 338679, 339666), the Juho Vainio Foundation, the Yrjö Jahnsson foundation, Päivikki and Sakari Sohlberg foundation, the Finnish Cultural Foundation. J.P.: reports support for the present

manuscript: EU, Academy of Finland and smaller grants from Finnish foundations supporting medical research (Juho Vainio Foundation, Päivikki and Sakari Sohlberg foundation, Finnish cultural foundation. E.v.M.: reports support for the present manuscript: Funding PASTURE study, FORALLVENT study, EFRAIM study and Leibniz prize; Grants: Bavarian State Ministry of Health and Care for “URS Study”, 01/21, German Federal Ministry of Education and Research (BMBF) *Deutsches Zentrum für Lungenforschung [German Center for Lung Research],* 01/21, Forschungsvorhaben: Rolle der mikrobiellen Umweltexposition für den Schutz vor Heuschnupfen und Asthma Research project: The role of the microbial environmental exposure for the protection against hay fever and asthma *Bayerisches Staatsministerium für Gesundheit und Pflege Bavarian State Ministry of Health and Care,* 01/20; Royalties or licenses: Elsevier GmbH, Georg Thieme Verlag, Springer-Verlag GmbH, Elsevier Ltd., Springer Nature Group; Consulting fees: Chinese University of Hongkong, European Commission, HiPP GmbH & Co KG, AstraZeneca, Imperial College London, OM Pharma, ALK-Abello Arzneimittel GmbH; Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events: Massachusetts Medical Society, Springer-Verlag GmbH, Elsevier Ltd., Böhringer Ingelheim International GmbH, European Respiratory Society (ERS), Universiteit Utrecht, Faculteit Diergeneeskunde, Universität Salzburg, Springer Medizin Verlag GmbH, Japanese Society of Pediatric Allergy and Clinical Immunology (JSPACI), Klinikum Rechts der Isar, University of Colorado, Paul-Martini-Stiftung, Astra Zeneca, Imperial College London, Children´s Hospital Research Institute of Manitoba, Kompetenzzentrum für Ernährung (Kern), OM Pharma S.A., Swedish Pediatric Society for Allergy and Lung Medicine, Chinese

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College of Allergy and Asthma (CCAA), ALK-Abello Arzneimittel GmbH, Abbott Laboratories, Deutscher Apotheker Verlag GmbH & Co. KG; Support for attending meetings and/or travel: Verein zur Förderung der Pneumologie am Krankenhaus Großhansdorf e.V., Pneumologie Developpement, Mondial Congress & Events GmbH & Co. KG, American Academy of Allergy, Asthma & Immunology, Imperial College London, Margaux Orange, Volkswagen Stiftung, Böhringer Ingelheim International GmbH, European Respiratory Society (ERS), Universiteit Utrecht, Faculteit Diergeneeskunde, Österreichische Gesellschaft f. Allergologie u. Immunologie, Massachusetts Medical Society, OM Pharma S. A., Hanson Wade Ltd., iKOMM GmbH, DSI Dansk Borneastma Center, American Thoracic Society, HiPP GmbH & Co KG, Universiteit Utrecht, Faculteit Bètawetenschappen; Patents planned, issued or pending: EvM has patent No. PCT/EP2019/085016 (Barn dust extract for the prevention and treatment of diseases) pending (Barn dust extract for the prevention and treatment of diseases) pending, royalties paid to ProtectImmun for patent EP2361632 (Specific environmental bacteria for the protection from and/or the treatment of allergic, chronic inflammatory and/or autoimmune disorders, granted on 19 March 2014), and patents EP1411977 (Composition containing bacterial antigens used for the prophylaxis and the treatment of allergic diseases, granted on 18 April 2007), EP1637147 (Stable dust extract for allergy protection, granted on 10 December 2008), and EP 1964570 (Pharmaceutical compound to protect against allergies and inflammatory diseases, granted on 21 November 2012) licensed to ProtectImmun. Patent EP21189353.2. 2021. von Mutius E, Rankl B, Bracher F, Müller C, Walker A, Hauck SM, Merl-Pham J, inventors; Proteins identified from bran dust extract for the prevention and treatment of

diseases. Patent PCT/US2021/016918. 2021. Martinez FD, Vercelli D, Snyder SA, von Mutius E, Pivniouk V, Marques dos Santos M, inventors; Therapetic fractions and proteins from athma-protective farm dust; Participation on a Data Safety Monitoring Board or Advisory Board: Member of the EXPANSE (funded by European Commission) Scientific Advisory Board, Member of the BEAMS External Scientific Advisory Board (ESAB), Member of the Editorial Board of “The Journal of Allergy and Clinical Immunology: In Practice”, Member of the Scientific Advisory Board of the Children’s Respiratory and Environmental Workgroup (CREW), Member of the International Scientific & Societal Advisory Board (ISSAB) of Utrecht Life Sciences (ULS), University of Utrecht, Member of External Review Panel of the Faculty of Veterinary Science, University of Utrecht, Member of the Selection Committee for the Gottfried Wilhelm Leibniz Programme (DFG), Member of the International Advisory Board of Asthma UK Centre for Applied Research (AUKCAR), Member of the International Advisory Board of “The Lancet Respiratory Medicine”, Member of the Scientific Advisory Board of the CHILD (Canadian Healthy Infant Longitudinal Development) study, McMaster University, Hamilton, Canada, Asthma UK Centre for Applied Research, Pediatric Scientific Advisory Board Iceland. All other authors have no conflict of interest in relation to this work. J.G: reports payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from The journal Pediatric Allergy and Immunology (co-owned by Wiley and EAACI); personal honorarium for serving as Associate Editor. M.K: reports grants or contracts from European Union, German Ministry of Education and Research, German Research Foundation, Infectopharm; consulting fees from Bionorica, Sanofi, Novartis, Bencard; payment or honoraria for

lectures, presentations, speakers bureaus, manuscript writing or educational events from ERS, EAACI, ATS, Novartis, Glaxo, Chiesi, Sanofi, Nutricia, Hipp, Allergopharma; patents planned, issued or pending METHOD FOR TESTING A SUBJECT THOUGHT TO HAVE OR TO BE PREDISPOSED TO ASTHMA European patent application 5 EP07301135.5. Other co-authors declares no conflict of interests.

Abstract word count: ~~252~~239

Manuscript word count: ~~3269~~3650

Abstract

Background: An important 'window of opportunity' for early life exposures has been proposed for the development of atopic eczema and asthma.

Objective: ~~It is h~~However it is, unknown whether hay fever with a peak incidence around late school age to adolescence is similarly determined very early in life.

Methods: In the PASTURE birth cohort potentially relevant exposures such as farm milk consumption and exposure to animal sheds were assessed at multiple time points from infancy to age 10.5 years and classified by repeated measure latent class analyses (N=769). Fecal samples at age 2 and 12 months were sequenced by 16S rRNA. Hay fever was defined by parental reported symptoms and/or physician's diagnosis of hay fever in the last 12 months using questionnaires at age 10.5 years.~~, and for sensitivity analyses (SA) by adding inhalant sensitization to the definition.~~

Results: Farm children had half the risk of hay fever at age 10.5 years (adjusted odds-ratio (aOR) [95% CI]=0.50 [0.31; 0.79]) compared to non-farm children. While early life events such as gut microbiome richness at age 12 months (aOR=0.66 [0.46; 0.96]) and exposure to animal sheds in the first three years of life (aOR=0.26 [0.06; 1.15]) were determinants of hay fever, the continuous consumption of farm milk from infancy up-to school age was necessary to exert the protective effect (aOR=0.35 [0.17; 0.72])~~ and SA: aOR=0.41 [0.17; 0.97])~~.

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Conclusion: While early life events determine the risk of subsequent hay fever, continuous exposure is necessary to achieve protection. These findings argue against the notion that only early life exposures set long-lasting trajectories.

Highlight box:

1. What is already known about this topic?

The protective effects of early life farm exposures and gut microbiome composition on atopic diseases and asthma proposes an important window of opportunity.

2. What does this article add to our knowledge?

Early life farm exposures also determine risk of hay fever. However, continuous farm milk consumption is necessary for optimal prevention, thereby arguing against the notion of an early-determined trajectory governing later outcomes.

3. How does this study impact current management guidelines?

These results emphasize the preventive potential of continuously drinking unprocessed farm milk for hay fever protection, suggesting carrying out clinical trials to test microbiologically safe cow's milk for protection from hay fever.

~~Early life farm exposures determine risk of hay fever. However, continuous farm milk consumption up to school age is necessary for optimal prevention, thereby arguing against the notion of an early-determined trajectory governing later outcomes.~~

Keywords: Childhood, farm milk, farming, gut microbiome, hay fever, animal sheds.

Abbreviations:

PASTURE: Protection against Allergy--Study in Rural Environments

IgE: immunoglobulin E

SPT: skin prick test

RMLCA: repeated measure latent class analyses

q: quintile

aOR: adjusted odds ratio

95%CI: 95% confidence interval

IQR: interquartile range

Introduction

Hay fever is the most common allergic disease worldwide with a prevalence between 20-30% (1). The high prevalence has a vast impact on ~~socio-economic~~ several factors such as quality of life and high healthcare costs (2, 3). Numerous epidemiological studies have shown the protective effect of early life farm exposures and gut microbiome composition~~exposures~~ on asthma, atopy, atopic sensitization, and hay fever (4-11), thus, proposing an important 'window of opportunity' for early life farm exposures and gut microbiome ~~exposures~~ composition for the protection of atopic diseases and asthma. However, it is unknown whether hay fever with a peak incidence around late school age to adolescence is only determined very early in life or whether later exposure before the onset of disease matters most.

The protective "farm-effect" has been attributed to two factors; consumption of unprocessed cow's milk, subsequently termed 'farm milk' and exposure to animal sheds (12-16). Hence, the aim of these analyses is to study the temporal pattern of these protective exposures on hay fever development using the longitudinal data from the PASTURE study. Furthermore, the role of the gut microbiome was investigated.

Methods

Study design and population

PASTURE is a prospective birth cohort study started in 2002 and is conducted in children from rural areas of 5 European countries (Austria, Finland, France, Germany, and Switzerland) (17). The study was designed to evaluate risk and preventive factors for atopic diseases. The study was approved by the local research ethics committees in each country, and written informed consent were obtained from the children's parents. Pregnant women were invited to participate during their third trimester of pregnancy. ~~The women were then classified into farm and non-farm groups.~~ The children from the participating women were recruited at birth. Children of mothers living on family-run livestock farms at birth of the children were assigned to the farm group. The non-farm group included children of mothers from the same rural areas but not living on a farm (18). Information were obtained through questionnaires in interviews or self-administered questionnaires from mothers.

Definitions of outcome:

Hay fever was defined by parent reported symptoms (itchy, runny, or blocked nose without a cold accompanied by red itchy eyes) and/or a physician's diagnosis of hay fever in the last 12 months using questionnaires at age 10.5 years. Allergen specific IgE and skin prick test (SPT) were assessed at age 10.5 years (19). Inhalant sensitization was defined as at least one IgE specific to alder, birch, hazel, plantain, mugwort, alternaria, grass, rye, *Dermatophagoides pteronyssinus*, *Dermatophagoides farina,* cat, dog, or horse at levels ≥0.7IUml^{-1} or SPT (birch, grass, alternaria, *Dermatophagoides*

pteronyssinus, Dermatophagoides farinae, cat, or dog) ≥3mm. A more stringent definition of hay fever consisting of hay fever plus inhalant sensitization at 10.5 years was used in sensitivity analyses.

Assessment of exposures:

The child's consumption of any farm milk, pasteurized and homogenized milk subsequently termed "processed milk" consumption, and any exposure to animal sheds (cows, pigs, sheep, or horses) at time points 12, 18 months, 2, 3, 4, 5, 6, and 10.5 years were assessed. In addition, maternal any farm milk consumption and animal sheds exposure was assessed during pregnancy and infant's consumption of any farm milk, processed milk and exposure to animal sheds (month 4-12) were obtained on weekly basis by diary. The exposure to animal sheds was further dichotomized based on third quartile (17 weeks) weeks spent on animal sheds as a cut-off.

Avoidance of milk or milk products was assessed at the age of 12, 18 months, 2, 3, 4, 5, and 6 years. Additionally, information on ~~the amount~~frequency of farm milk consumption was assessed at the age of 18 months, 2, 3, 4, 5, 6 and 10.5 years of age. ~~Amount~~ Frequency of processed milk consumption was assessed at age 10.5 years.

DNA extraction from fecal samples and sequencing analyses:

Fecal samples were collected from children's diapers during the home visit at the age of 2 and 12 month. DNA was extracted from homogenized samples and bioinformatics processing were performed as previously described in detail (10). Briefly, α-diversity (i.e. richness and Shannon-index) was calculated as average of multiple times rarefied

samples (10). Metabolite levels of short chain fatty acids (SCFA) were measured in fecal samples obtained from 301 children at the age of 12 months (20, 21). Two variables, butyrate and propionate scores were created by modeling SCFA-levels on the relative abundance of all bacterial genera using random forest model in the R-package ranger.

Statistical analyses

We performed repeated measure latent class analyses (RMLCA) using data from pregnancy to age 10.5 years i.e. 9 time points were included separately for exposure to animal sheds, and farm milk consumption (Figure 1(a-b)). The children were allocated to specific exposure classes by their highest posterior probabilities. The analyses were done on children having data at least at 7 of the 9 assessed time points. The optimal number of exposure classes was then determined according to the Bayesian Information Criterion and the labelling of the exposure classes was based on main features of each class.

Further as sensitivity analyses, we repeated the farm milk RMLCA, in subgroup of children without a family history of parental asthma and/or atopy and excluding children avoiding milk or milk products at the age 1–6 years as it could introduce confounding by reverse causation, i.e. a positive family history. A farm milk consumption score (Methods section in the Online Repository Text) reflecting the ~~amount~~ frequency of farm milk consumed was built and divided into quintiles. The quintiles were further categorized as low (q1), intermediate (q2-q4) and high (q5).

The associations between hay fever and potential exposures (farm milk exposure classes, animal sheds exposure classes, ~~amount~~ frequency of farm milk consumption (continuous and quintiles), ~~amount~~ frequency of processed milk consumption, SCFAs (butyrate score and propionate score) as well as gut microbiome's richness, and Shannon-index) were assessed by logistic regression. We tested the differences in relative abundance of most common single bacterial genera at 2 and 12 months with hay fever by Wilcoxon test (10). The associations between gut microbiome richness and farm milk consumption, processed milk consumption and exposure to animal sheds during infancy was assessed by linear regression. The effect estimates are presented as adjusted odds ratios (aORs) for logistic regression and geometric mean ratios (GMR; calculated by exponentiation of the regression coefficients and their 95% confidence intervals (95%CI)) for linear regression along with their respective 95%CI and a *P value* of 0.05 was considered significant. The above models were adjusted for centers and ~~potential~~ confounders (growing up on a farm and parental asthma and/or atopy) associated with hay fever and exposures in our study. No other ~~potential~~ confounders i.e. associated with both outcome and exposures were found. We additionally calculated the Number Needed to Treat (NNT), which is the effectiveness of a treatment on an outcome using an R-script (22).

Furthermore, we conducted mediation analyses to assess whether the associations between farm milk consumption and exposure to animal sheds in infancy (4-12 months) and the risk of hay fever is mediated by gut microbiome features adjusting for centers. The mediation analysis was conducted through path analysis using maximum likelihood

test to estimate the regression parameters in Mplus 8.5 (23). The mediating effect is reported as the proportion of the estimated indirect effect to the total effect.

The statistical analyses were performed with SAS 9.4 software (SAS Institute, Cary, NC) and Mplus 8.5 software (Muthén & Muthén, Los Angeles, California).

Results

Characteristics of the study population

At 10.5 year follow up 778 children participated in the PASTURE study and 769 have data on hay fever. Comparing the baseline characteristics between included (N=769) and excluded children (N=364) did not show any significant difference except ~~of having contact to dogs at age 2 months~~for maternal age at pregnancy, maternal smoking, parental education, and premature birth (Table E1 Online Repository Text). ~~In the PASTURE birth cohort, d~~Data on farm milk consumption and exposure to animal sheds at least at one time point (from pregnancy, age of 12, 18 months, 2, 3, 4, 5, 6, and 10.5 years) was available for ~~962~~ all these children. Of these, 769 children had information on hay fever at 10.5 years of age. ~~Comparing the baseline characteristics between included and excluded children did not show any significant difference except of having contact to dogs at age 2 months (Table E1 Online Repository).~~ The proportion of children growing up on a farm was 47.7%. Hay fever at the age of 10.5 years was reported in 12.9% children. Of these, 28.9%, 36.7%, and 21.7% had asthma, eczema, and food allergy at age 10.5 years respectively (Table 1). Further, 86.8% were sensitized to inhalant allergens at age 10.5 years (Table 1). Figure E1 (Online Repository Text) shows the proportion of children who were consuming farm milk or were exposed to animal sheds at each time point. The consumption of farm milk by children increased from the age of 1 to 3 years and gradually decreased after age 4 years. Similarly, exposure to animal sheds also increased from the age of 1 to 4 years and slightly decreased after age 5 years.

Temporal pattern of the farm-related exposures on hay fever

Children growing up on a farm had half the risk of hay fever as compared to non-farm children (aOR [95%CI], *P value*: 0.50 [0.31; 0.79], *0.003*).

In a first step, we analyzed the temporal pattern of exposure to animal sheds ('continuous exposure to animal sheds', 'only early exposure to animal sheds', 'only late exposure to animal sheds' and 'no exposure to animal sheds'; Figure 1(a)) on hay fever development. Of these categories, 'only early exposure to animal sheds' showed an inverse association when compared to 'no exposure to animal sheds' which however did not reach statistical significance (0.26 [0.06; 1.15], *0.08*) (Table E2 Online Repository Text). When adjusting this model for consumption of farm milk exposure classes, the results remained unchanged (Table E2 Online Repository Text).

We then analyzed the temporal pattern of consumption of farm milk in similar categories 'continuous consumption of farm milk', 'only early consumption of farm milk', 'only late consumption of farm milk' and 'no consumption of farm milk' (Figure 1(b)). The strongest inverse association was found for the 'continuous consumption of farm milk' as compared to 'no consumption of farm milk' (0.35 [0.17; 0.72], *0.004*) exposure class (Figure 2 and Table E3 Online Repository Text). In contrast, 'only early consumption of farm milk' showed no significant effect on hay fever. The inverse association of 'continuous consumption of farm milk' compared to 'no consumption of farm milk' was still observed when using the stringent definition of hay fever (0.41 [0.17; 0.97], *0.04*) (Figure E2 Online Repository Text) or incident hay fever at age 10.5 years (0.39 [0.15;

0.99], *0.05,* data not shown). ~~Furthermore, stratification by center yielded similar effects, thus replicating the findings in the independent PASTURE populations (Table E4 Online Repository).~~ Since confounding by reverse causation might have biased our findings, we ran a sensitivity analysis in the subgroup of children without a family history of parental asthma and/or atopy and excluded children avoiding milk or milk products at the age 1–6 years. This did not change the inverse association with hay fever (~~Table E4 Online Repository~~0.21 [0.06; 0.78], *0.02*, data not shown).

We next assessed the association of the ~~amount~~ frequency of farm milk consumption i.e. whether frequently drinking ~~more~~ farm milk has a dose-response effect on hay fever. The highest compared to the lowest quintile of farm milk consumption was inversely associated with hay fever (0.37 [0.16; 0.84], *0.02*), whereas the intermediate group (q2-q4; 0.63 [0.37; 1.10], 0.10) showed a similarly inverse but non-significant association ~~(Table E5 Online Repository)~~. Similar results were obtained when using ~~amount~~ frequency of farm milk consumption score as a continuous variable (data not shown).

We further investigated if consumption of processed milk shows similar effects as consumption of farm milk (Figure E3(a) Online Repository Text). Consumption of 'high farm and low processed milk' was inversely associated with hay fever (0.24 [0.09; 0.66], *0.006)*, however, the consumption of processed milk attenuated the farm milk effect when both farm milk and processed milk were consumed ('mixed consumption of farm and processed milk' (0.43 [0.19; 0.96], *0.04)* (Figure E3(b) and Table E3 Online Repository Text). Furthermore, daily consumption of shop milk at the age of 10.5 years showed association in positive direction with hay fever (Figure E4 Online Repository Text).

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Additionally, NNT calculated in our study was 7.14, i.e. 7 children would have to drink farm milk continuously from pregnancy by mothers until age 10.5 years in order to prevent hay fever in one child.

Early life effect of gut microbiome on hay fever

We investigated the role of the early life gut microbiome by relating bacterial composition, richness, Shannon-index (at age 2 and 12 months) and SCFA to hay fever.

We did not find any significant differences in relative abundance of most common bacterial genera at 2 and 12 months with subsequent hay fever at 10.5 year (data not shown). Also, richness and Shannon-index of bacteria at 2 months were not associated with hay fever at 10.5 years (Figure 3). However, the bacterial richness of the gut microbiome at 12 months was inversely associated with hay fever (aOR [95%CI], *P value*: 0.66 [0.46; 0.96], *0.03,* Figure 3). Shannon-index at 12 months also showed an inverse non-significant trend for hay fever (0.71 [0.49; 1.04], *0.08,* Figure 3). The SCFAs butyrate (1.00 [0.92; 1.09], *0.99)* and propionate scores (0.97 [0.90; 1.05], *0.50)* were in turn not associated with hay fever (data not shown) ~~(Figure E5)~~. We reasoned that consumption of milk and exposure to animal sheds may shape the gut microbiome, in particular its richness. Consumption of farm milk (aGMR [95%CI]: 1.20 [1.03; 1.40], *P value=0.02*) and exposure to animal sheds (aGMR [95%CI]: 1.19 [1.01; 1.40], *P value=0.04*) in the first year of life increased gut microbiome richness (Figure 4). In turn, no association was observed for consumption of processed milk (Figure 4). Since both, farm milk consumption and exposure to animal sheds during infancy (4-12 months) showed significant associations with gut microbiome richness at 12 months, we

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performed a mediation analysis including unexposed and children exposed to both in infancy. The mediation analysis revealed that part (18.4%) of the total protective effect of farm milk consumption and exposure to animal sheds in the first year of life on hay fever was mediated by gut microbiome richness (*P value=0.03*, Figure 5). The number of children only being exposed to animal sheds or farm milk, respectively, was too low to allow separate mediation analyses.

Discussion

In the PASTURE birth cohort, the continuous consumption of farm milk throughout age 10.5 years, but neither the only early nor the only late exposure alone was significantly associated with reduced risk of hay fever at age 10.5 years. In contrast, exposure to animal sheds only exerted a trend towards protection early in life. Both exposures, farm milk and animal sheds, early in life increased gut microbiome richness at age 12 months, which partly explained the protective effect of these exposures on hay fever.

The human gut microbiome composition plays an important role in shaping the development of the immune system (24). There is some evidence that the gut microbiome diversity in the first years of life may protect from atopic sensitization. In the population based CHILD cohort, the Shannon-index at age 3 months was associated with protection from atopic sensitization at 1 year (8). However, in a Swedish study the Shannon-index in early infancy was not associated with allergic rhinoconjunctivitis and SPT at age 7 years (25). Our analyses likewise do not confirm this very early 'window of opportunity' since gut microbiome richness and Shannon-index at age 2 month was unrelated to hay fever development.

In contrast, gut microbiome richness at the age of 1 year was inversely associated with hay fever at age 10.5 years. We have previously shown in the PASTURE cohort in agreement with others that the compositional structure of the gut microbiome undergoes very significant changes from early age when most infants are breastfed to age 12 months ~~and~~ when most foods have been introduced into a child's diet (10, 11). Nevertheless, an inverse association of gut microbiome richness at age 1 year with an

outcome much later in life at age 10.5 years may seem surprising. This long-term association may be attributable to an earlier onset of disease. In fact, ~~9.2~~4.6%, 5.9% and ~~15.6~~6.7% of children with data on hay fever at age 10.5 years had already reported symptoms and/or a diagnosis of hay fever at age 4, 5 and ~~5~~ 6 years, respectively. Furthermore, early alterations of the composition of the gut microbiome may shape its subsequent development towards an adult-like compositional structure in the first 3 years of life (26). Unfortunately, no fecal samples have been collected at later time points in the PASTURE cohort.

The production of the SCFAs butyrate and propionate measured at 12 months of age has been reported previously as determinants of protection against atopic sensitization at age 6 years (20). In our study, no relation between the SCFAs butyrate and propionate with hay fever was found. Furthermore, no association with single taxa was seen. Thus, different facets of the early development of the gut microbiome composition may matter for different clinical outcomes.

Of the environmental exposures investigated in these analyses, the continuous, but neither the early nor the late, consumption of farm milk was seen to protect from hay fever development. Moreover, a dose-response effect was found corroborating the strength of the observation. Interestingly, this protective effect was partly mediated by gut microbiome richness which may suggest that a continued exposure to unprocessed cow's milk may increase gut microbiome richness beyond the age of 12 months and thereby confer ~~it's~~ its protective effect.

Continuous exposure also implies repeated exposures. The novel concept of trained immunity may lend itself to mechanistic speculations since phenomena like LPS tolerance are based on the necessity of repeated rather than single exposures (27).
A potential explanation for the differential effect of unprocessed versus processed cow's milk is grounded in the observation that most farm children drink their milk unboiled. In fact, too few children received only boiled, i.e. heat treated farm milk over the study period to allow meaningful stratified analyses. A number of population-based and experimental studies have stressed the potential importance of heat-treatment of cow's milk for the loss of protective effects (16, 28-31). Whether alterations of the milk microbiome or denaturation and loss of function of milk (whey) proteins underlie these findings awaits further elucidation.

Exposure to animal sheds during early years showed an inverse, albeit non-significant effect on hay fever. This is in contrast to previous farm studies showing stronger effects (12, 32). The discrepancy might be attributable to important differences in the definition of exposure to animal sheds used in the PASTURE study, which only assessed exposure to any animal sheds without differentiating between cows, pigs, sheep and horses. The nature of animal exposure may however matter. While exposure to cow sheds showed a significant protective effect on hay fever and asthma (12), sheep sheds and keeping of hares and rabbits were risk factors for wheezing and asthma respectively in the PARSIFAL farm study (33).
The main strength of this study is its longitudinal design, which enabled us to assess the exposures at several time points before the assessment of the outcome. ~~The study population comes from five European countries; however, the consumption of farm milk~~

~~effect was replicable in all the PASTURE centers, thus substantiating the observations.~~ ~~Likewise, o~~Excluding children with parental asthma and/or atopy and who were avoiding milk or milk products showed similar inverse associations with hay fever consequently arguing against confounding by reverse causation. An elevated risk of diarrhea and farm milk consumption at 10.5 years was not observed (data not shown). The results of the present study show protective association of continuous consumption farm milk on hay fever. However, one of the potential caveats of the observation study is finding causality. Hence, the Milk Against Respiratory Tract Infections and Asthma (MARTHA) an ongoing interventional trial is being carried out to evaluate the preventive effect of minimally treated, i.e. only pasteurized and thus microbiologically safe cow's milk on upper respiratory tract infections and allergy (34). Further, the NNT in our study was 7, however, this study is not a randomized placebo-controlled double-blind trail and thus numbers must be taken with some caution. One of the drawbacks of the study is the missing data on hay fever at 10.5 years. However, comparing the baseline characteristics between included and excluded children did not show any significant difference except ~~of~~ for maternal age at pregnancy, maternal smoking, parental education, and premature birth~~having contact to dogs at age 2 months~~. However, adjusting for these variables ~~analyses for contact to dogs~~ did not change the results (data not shown). Another drawback is the small number in the "only early" and "only late" exposure groups that shows protective non-statistical significant effect on hay fever. However, using the RMLCA approach our study could identify these small groups manifesting that these types of habits i.e. farm milk consumption or exposure to animal sheds do exist. We performed a posthoc power calculation using SAS and considering

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α=0.05 (two-sided). For our sample size of 650, i.e. in the exposure groups 'continuous consumption of farm milk' and 'no consumption of farm milk' the power of study is over 80% assuming the response probabilities ranging from 0.02-0.18 for having hay fever in children who consume farm milk and unadjusted OR of 0.24. Thus, our study was well powered to detect a relatively strong effect of farm milk consumption on hay fever.

In summary, the results of the present study demonstrate that continuous exposure of the main determinant, i.e. farm milk consumption but neither only early nor only late exposure alone conferred protection from hay fever development. The early compositional structure of the gut microbiome at age 1 year, but not age 2 month, did however in part mediate this protective effect. One might speculate that continuous consumption of unprocessed cow's milk may also increase gut microbiome richness at later ages, but we do not have data to support this notion. Overall, the findings presented herein do not support the notion of an early-determined trajectory where only early exposures in the first months of life would govern later outcomes. These results emphasize the preventive potential of continuously drinking unprocessed farm milk for hay fever protection. However, the risks associated with raw cow's milk consumption prohibit its recommendation for daily life. The results of the MARTHA trial however will shed light on potential side effects (34). Further clinical trials based on the present results are warranted.

Acknowledgments:

~~* The members of the PASTURE study group are Johanna Theodorou (Dr. von Hauner Children's Hospital, Ludwig Maximilians University Munich, Munich, Germany; Member of the German Center for Lung Research, DZL, Germany), Andreas Böck (Dr. von Hauner Children's Hospital, Ludwig Maximilians University Munich, Munich, Germany), Harald Renz (Institute of Laboratory Medicine, Philipps University of Marburg, Marburg, Germany; Department of Clinical Immunology and Allergology, Laboratory of Immunopathology, Sechenov University, Moscow, Russia), Petra I. Pfefferle (Comprehensive Biobank Marburg CBBM, Fachbereich Medizin der Philipps Universität Marburg, Marburg, Germany), Jon Genuneit (Pediatric Epidemiology, Medical Faculty, Leipzig University, Germany), Michael Kabesch (Department of Pediatric Pneumology and Allergy, University Children's Hospital Regensburg (KUNO) at the Hospital St. Hedwig of the Order of St. John, University of Regensburg, Regensburg, Germany), Marjut Roponen (Department of Environmental and Biological Sciences, University of Eastern Finland, Kuopio, Finland), and Lucie Laurent (University of Besançon, Department of Respiratory Disease, UMR/CNRS6249 Chrono-environment, University Hospital, Besançon, France).~~ We thank David A. Mills, Diana Taft and Karen Kalanetra from the Department of Food Science & Technology, University of California, Davis, USA for their contribution in the generation of the original microbiome sequence and analysis.

1. World Allergy Organization (WAO): World Allergy Week 2016
Pollen Allergies – Adapting to a Changing Climate
2. Tkacz JP, Rance K, Waddell D, Aagren M, Hammerby E. Real-world evidence costs of allergic rhinitis and allergy immunotherapy in the commercially insured United States population. Curr Med Res Opin. 2021;37(6):957-65.
3. Zuberbier T, Lotvall J, Simoens S, Subramanian SV, Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2) LEN review. Allergy. 2014;69(10):1275-9.
4. von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. Nat Rev Immunol. 2010;10(12):861-8.
5. Genuneit J. Exposure to farming environments in childhood and asthma and wheeze in rural populations: a systematic review with meta-analysis. Pediatr Allergy Immunol. 2012;23(6):509-18.
6. Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, Jenmalm MC. Low diversity of the gut microbiota in infants with atopic eczema. J Allergy Clin Immunol. 2012;129(2):434-40, 40 e1-2.
7. Azad MB, Konya T, Guttman DS, Field CJ, Sears MR, HayGlass KT, et al. Infant gut microbiota and food sensitization: associations in the first year of life. Clin Exp Allergy. 2015;45(3):632-43.
8. Boutin RCT, Sbihi H, Dsouza M, Malhotra R, Petersen C, Dai D, et al. Mining the infant gut microbiota for therapeutic targets against atopic disease. Allergy. 2020;75(8):2065-8.
9. Chen CC, Chen KJ, Kong MS, Chang HJ, Huang JL. Alterations in the gut microbiotas of children with food sensitization in early life. Pediatr Allergy Immunol. 2016;27(3):254-62.
10. Depner M, Taft DH, Kirjavainen PV, Kalanetra KM, Karvonen AM, Peschel S, et al. Maturation of the gut microbiome during the first year of life contributes to the protective farm effect on childhood asthma. Nat Med. 2020;26(11):1766-75.
11. Stokholm J, Blaser MJ, Thorsen J, Rasmussen MA, Waage J, Vinding RK, et al. Maturation of the gut microbiome and risk of asthma in childhood. Nat Commun. 2018;9(1):141.
12. Illi S, Depner M, Genuneit J, Horak E, Loss G, Strunz-Lehner C, et al. Protection from childhood asthma and allergy in Alpine farm environments-the GABRIEL Advanced Studies. J Allergy Clin Immunol. 2012;129(6):1470-7 e6.
13. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, et al. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. J Allergy Clin Immunol. 2011;128(4):766-73 e4.
14. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, et al. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. Clin Exp Allergy. 2007;37(5):661-70.
15. Braun-Fahrlander C, von Mutius E. Can farm milk consumption prevent allergic diseases? Clin Exp Allergy. 2011;41(1):29-35.
16. Loss G, Depner M, Ulfman LH, van Neerven RJ, Hose AJ, Genuneit J, et al. Consumption of unprocessed cow's milk protects infants from common respiratory infections. J Allergy Clin Immunol. 2015;135(1):56-62.
17. von Mutius E, Schmid S, Group PS. The PASTURE project: EU support for the improvement of knowledge about risk factors and preventive factors for atopy in Europe. Allergy. 2006;61(4):407-13.

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18. Hose AJ, Depner M, Illi S, Lau S, Keil T, Wahn U, et al. Latent class analysis reveals clinically relevant atopy phenotypes in 2 birth cohorts. J Allergy Clin Immunol. 2017;139(6):1935-45 e12.
19. Chauveau A, Dalphin ML, Mauny F, Kaulek V, Schmausser-Hechfellner E, Renz H, et al. Skin prick tests and specific IgE in 10-year-old children: Agreement and association with allergic diseases. Allergy. 2017;72(9):1365-73.
20. Roduit C, Frei R, Ferstl R, Loeliger S, Westermann P, Rhyner C, et al. High levels of butyrate and propionate in early life are associated with protection against atopy. Allergy. 2019;74(4):799-809.
21. Dostal A, Baumgartner J, Riesen N, Chassard C, Smuts CM, Zimmermann MB, et al. Effects of iron supplementation on dominant bacterial groups in the gut, faecal SCFA and gut inflammation: a randomised, placebo-controlled intervention trial in South African children. Br J Nutr. 2014;112(4):547-56.
22. A P. How to calculate the Number Needed to Treat (NNT) from Cohen's d or Hedges'g. Retrieved from https://rpubs.com/RatherBit. 2015.
23. Muthén LKM, B.O. Mplus User's Guide 7th edition (Muthén & Muthén, 1998-2012).
24. Tanaka M, Nakayama J. Development of the gut microbiota in infancy and its impact on health in later life. Allergol Int. 2017;66(4):515-22.
25. Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, Jenmalm MC. Low gut microbiota diversity in early infancy precedes asthma at school age. Clin Exp Allergy. 2014;44(6):842-50.
26. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 2012;486(7402):222-7.
27. Netea MG, Dominguez-Andres J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. Nat Rev Immunol. 2020;20(6):375-88.
28. Abbring S, Verheijden KAT, Diks MAP, Leusink-Muis A, Hols G, Baars T, et al. Raw Cow's Milk Prevents the Development of Airway Inflammation in a Murine House Dust Mite-Induced Asthma Model. Front Immunol. 2017;8:1045.
29. Abbring S, Wolf J, Ayechu-Muruzabal V, Diks MAP, Alhamwe BA, Alhamdan F, et al. Raw Cow's Milk Reduces Allergic Symptoms in a Murine Model for Food Allergy-A Potential Role For Epigenetic Modifications. Nutrients. 2019;11(8).
30. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, et al. omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. J Allergy Clin Immunol. 2016;137(6):1699-706 e13.
31. Sozanska B. Raw Cow's Milk and Its Protective Effect on Allergies and Asthma. Nutrients. 2019;11(2).
32. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet. 2001;358(9288):1129-33.
33. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, et al. Not all farming environments protect against the development of asthma and wheeze in children. J Allergy Clin Immunol. 2007;119(5):1140-7.
34. Brick T, Hettinga K, Kirchner B, Pfaffl MW, Ege MJ. The Beneficial Effect of Farm Milk Consumption on Asthma, Allergies, and Infections: From Meta-Analysis of Evidence to Clinical Trial. J Allergy Clin Immunol Pract. 2020;8(3):878-89 e3.

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Figure legends

Figure 1. Types of exposure classes.

Solution for repeated measure latent classes defined by different exposures, which are a) exposure to animal sheds, and b) farm milk consumption in the PASTURE children. Numbers in parentheses indicate the total number of children in each class.

Figure 2. Associations of farm milk exposure classes with hay fever at age 10.5 years.

Associations of farm milk exposure classes with hay fever at age 10.5 years. Models are adjusted for centers, growing up on a farm, and parental atopy. The forest plot represent the adjusted odds ratios (aOR) with 95%confidence intervals [95%CI].

Figure 3. Association of gut microbiome richness, and Shannon-index at the age of 2 and 12 months with hay fever at 10.5 years.

Association of gut microbiome richness, and Shannon-index at months 2 (hay fever/total: 59/439) and 12 (hay fever/total: 79/633) with hay fever at 10.5 years. Models are adjusted for

centers, growing up on a farm, and parental atopy. The association with hay fever is shown as aOR per-interquartile-range of the probability along with 95%CI.

Figure 4. Association of consumption of farm milk, consumption of processed milk, and exposure to animal sheds in infancy with gut microbiome richness at month 12.

Association of consumption of farm milk (N=624), consumption of processed milk (N=624) and exposure to animal sheds (N=617) with richness at 12 months. Models are adjusted for centers, growing up on a farm, and parental atopy. The forest plot represent the adjusted geometric mean ratios with 95%CI.

Figure 5. Mediation analysis.

Mediation analysis of the protective effect of consumption of farm milk and exposure to animal sheds in infancy on hay fever mediated by gut microbiome richness at 12 months adjusting for centers (N=466). The figure shows the direct (β_1), indirect (β_2) and total (β) effects as well as their respective 95% CI from the path model. The proportion of the mediated (indirect) effect was 18.4%.

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699 **Table 1:** Description of the study population

Characteristic	All (N=769)	Hay fever (N=99 (12.9%))	No hay fever (N=670 (87.1%))	*P value*
	N (%)/Total	N (%)/Total	N (%)/Total	
Farm child (yes)	367 (47.7)/768	31 (31.3)/99	336 (50.2)/670	*0.0005*
Exposure to cats at age of 2 months (yes)	199 (26.0)/767	19 (19.2)/99	180 (27.0)/668	*0.11*
Exposure to dogs at age of 2 months (yes)	147 (19.2)/766	17 (17.2)/99	130 (19.5)/667	*0.68*
Maternal age at pregnancy (years) †	31.2±4.5 (N=769)	31.4±4.4 (N=99)	31.2±4.5 (N=670)	*0.52*
Maternal smoking (yes)	96 (12.5)/766	16 (16.5)/97	80 (12.0)/669	*0.25*
Second hand smoking (yes)	33 (4.3)/764	3 (3.1)/98	30 (4.5)/666	*0.79*
Parental education (yes)				*0.13*
Low	62 (8.1)/764	3 (3.1)/97	59 (8.9)/667	
Medium	280 (36.7/764)	39 (40.2)/97	241 (36.1)/667	
High	422 (56.7)/764	55 (56.7)/97	367 (55.0)/667	
Use of antibiotics during pregnancy (yes)	204 (27.0)/755	26 (26.5)/98	178 (27.1)/657	*1.00*

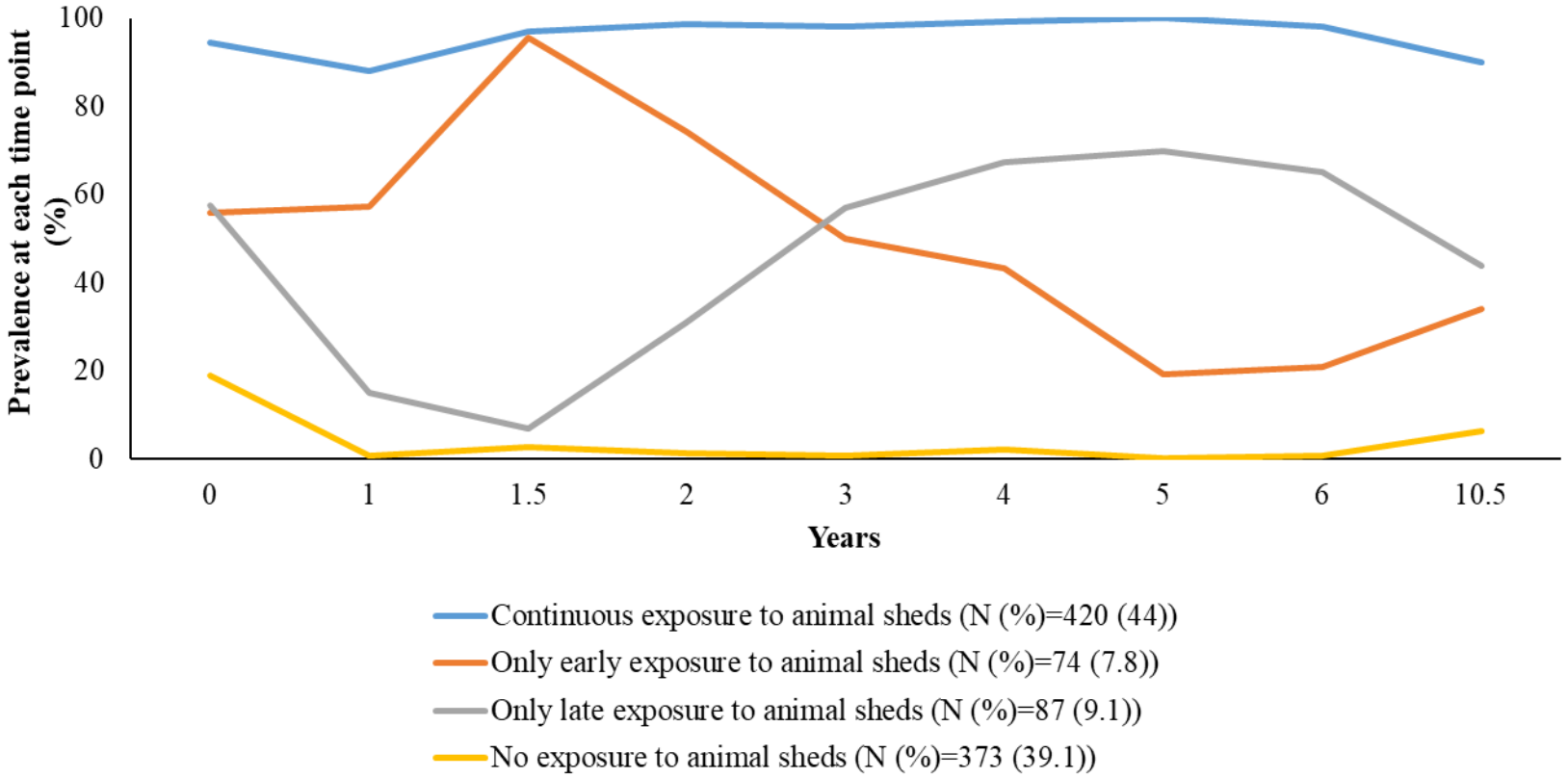
Parental atopy (yes)	416 (54.4)/765	72 (73.5)/98	344 (51.6)/667	*<0.0001*
Mode of delivery (normal)	624 (81.9)/762	82 (83.7)/98	542 (81.6)/664	*0.68*
Premature birth (yes)	11 (1.4)/769	1 (1.0)/99	10 (1.5)/670	*1.00*
Birth weight (kg) †	3.4±0.44 (N=605)	3.4±0.5 (N=82)	3.4±0.4 (N=523)	*0.81*
Breast feeding 2 months (yes)	711 (92.7)/767	90 (90.9)/99	621 (93.0)/668	*0.41*
Gender (female)	366 (47.7)/768	42 (42.4)/99	324 (48.4)/669	*0.28*
Having siblings (yes)	494 (64.2)/769	60 (60.6)/99	434 (64.8)/670	*0.43*
Use of antibiotics during first year of life (weeks) †	0.03±0.3 (N=746)	0.01±0.1 (N=97)	0.03±0.4 (N=649)	*0.86*
Doctor‘s diagnosis of hay fever (yes)	36 (4.7)/769	36 (36.4)/99	NA	*NA*
Inhalant sensitization (IgE≥0.7 kU/L or SPT≥3mm) at 10.5 years	259 (49.6)/522 ~~/522*~~	66 (86.8)/-76*	193 (43.3)/446*	*<0.0001*
Concomitants				
Asthma (yes)	69 (9.0)/764	28 (28.9)/97	41 (6.2)/667	*<0.0001*
Eczema (yes)	100 (13.1)/763	36 (36.7)/98	64 (9.6)/665	*<0.0001*
Food allergy (yes)	41 (5.5)/746	21 (21.7)/97	20 (3.1) /649	*<0.0001*

The categorical variables are presented as frequency (percentage) and the continuous variables as ~~†~~mean †: mean±standard deviation~~: median (quartile 1; quartile 3)~~. The test for differences between the groups are $\chi 2$ or Fischer's Exact test for categorical variables and Mann Whitney U test for continuous variables.

Farm child was defined as "Children of mothers living on family-run livestock farms were assigned to the farm group. The non-farm group included children of mothers from the same rural areas but not living on a farm". Exposure to pets at the age of 2 months (cats and dogs) was defined by asking "if you have cats?", "if you have dogs?" and "if they stay indoors in the house?~~"~~" . Maternal smoking during pregnancy was defined using the following questions "Have you in your life smoked more than 5 packs of cigarettes?" Or "Have you quit smoking in the meantime?" and if yes "Was it during this pregnancy?". Smoking by father, "Have you in your life smoked more than 5 packs of cigarettes?" Or "Do you still smoke?". Second hand smoking "How many cigarettes are on average per day were smoked in your house by other people?" If greater than 1 then second hand smoking was defined as 1 else 0. Parental education was defined as low (less than 10 years), medium (10 years) and high (greater than 10 years). Parental atopy was defined as doctor's diagnosis of hay fever, atopic dermatitis, or asthma ever in mother or father. Use of antibiotics during pregnancy was defined by asking "Have you taken antibiotics since the beginning of pregnancy?" Or "Have you taken any antibiotics during this pregnancy?". Child was defined as premature if the child was born before the completion of 37 weeks of pregnancy. Use of antibiotics by a child during first year of life was defined as "Total No. of weeks with antibiotics ingested". Breastfeeding at the age of 2 months (yes or no) was defined by asking "if you have ever breastfed?". ~~Parental atopy was defined as doctor's diagnosis of hay fever, atopic dermatitis, or asthma ever in mother or father.~~ SPT: skin prick test. Inhalant sensitization was defined as at least one IgE specific to alder, birch, hazel, plantain, mugwort, alternaria, grass, rye, *Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, *cat*, *dog*, *or horse* at levels $\geq 0.7 IUml^{-1}$ or SPT (birch, grass, alternaria, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, or dog) ≥3mm. Serum specific IgE and SPT was not measured in the Austrian study center, hence only sub-sample N=522 was included. ~~* sensitized to inhalant allergens/total.~~ Asthma was defined as a physician's diagnosis of asthma or recurrent obstructive bronchitis established until 10.5 years. Eczema and food allergy were defined as physician diagnoses at least once until the age of 10.5 years. NA: not applicable.

Figure 1.

a)


Prevalence at each time point (%)
100
80
60
40
20
0
0
1
1.5
2
3
4
5
6
10.5
Years
Continuous exposure to animal sheds (N (%)=420 (44))
Only early exposure to animal sheds (N (%)=74 (7.8))
Only late exposure to animal sheds (N (%)=87 (9.1))
No exposure to animal sheds (N (%)=373 (39.1))

b)

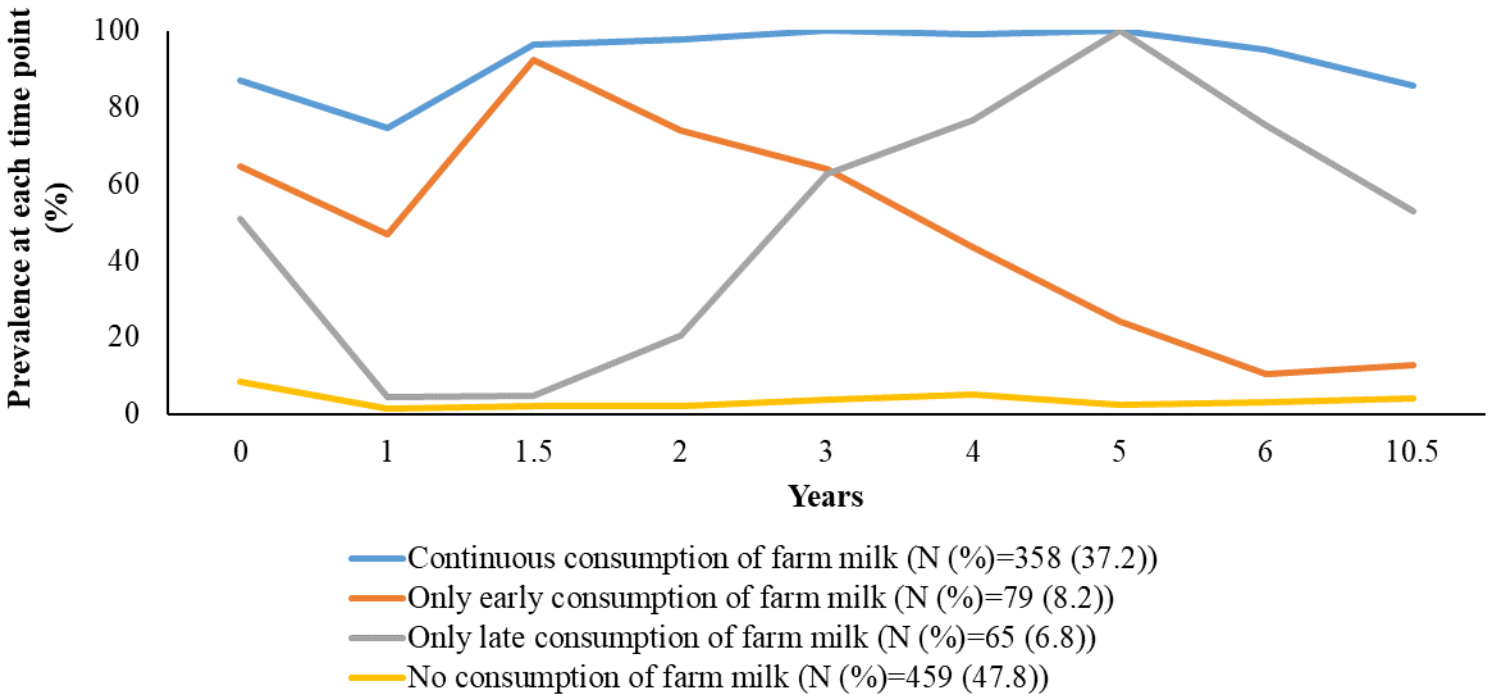

Prevalence at each time point (%)
100
80
60
40
20
0
0
1
1.5
2
3
4
5
6
10.5
Years
Continuous consumption of farm milk (N (%)=358 (37.2))
Only early consumption of farm milk (N (%)=79 (8.2))
Only late consumption of farm milk (N (%)=65 (6.8))
No consumption of farm milk (N (%)=459 (47.8))

Figure 2

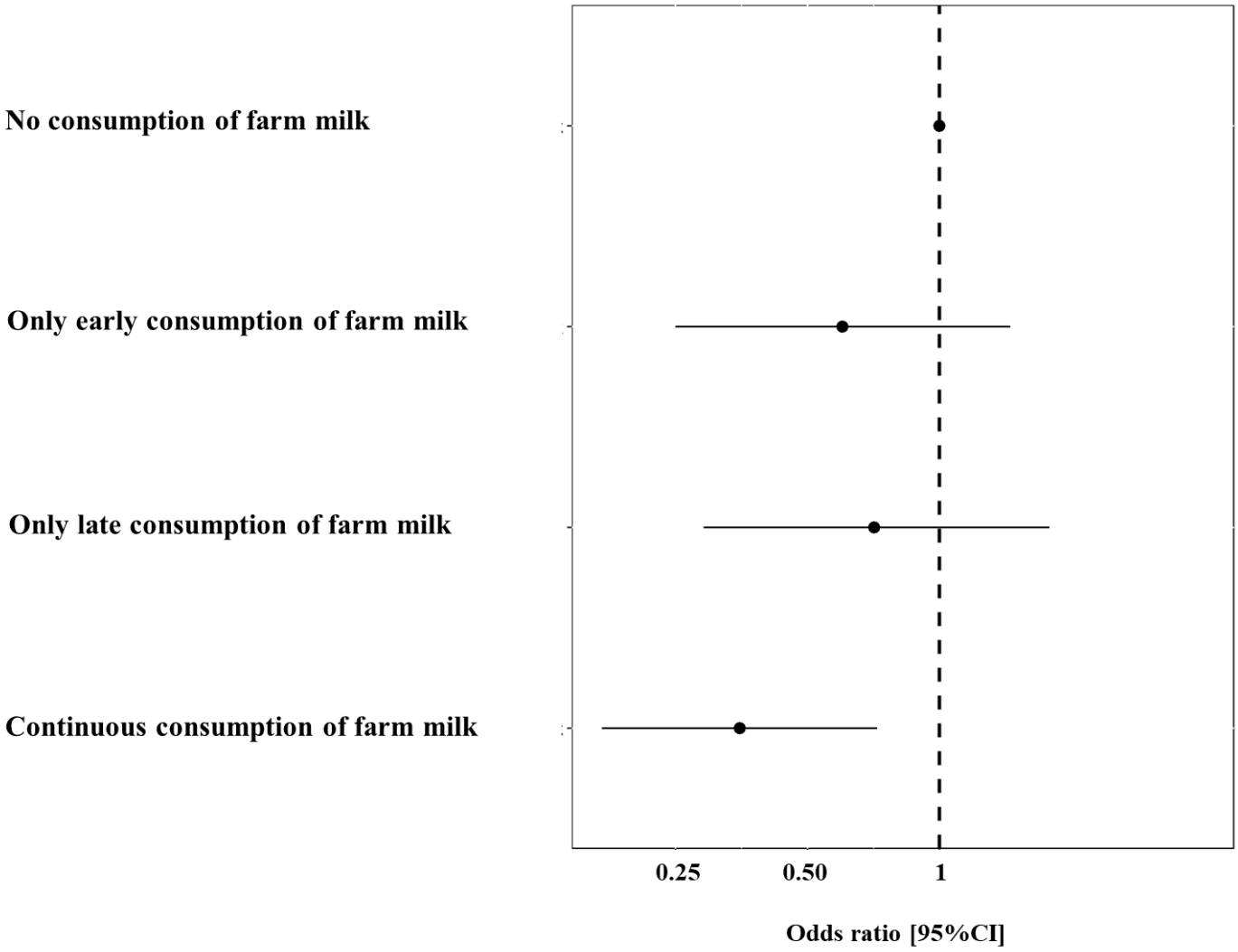

No consumption of farm milk
Only early consumption of farm milk
Only late consumption of farm milk
Continuous consumption of farm milk
0.25
0.50
1
Odds ratio [95%CI]

Figure No. - 3

Figure 3

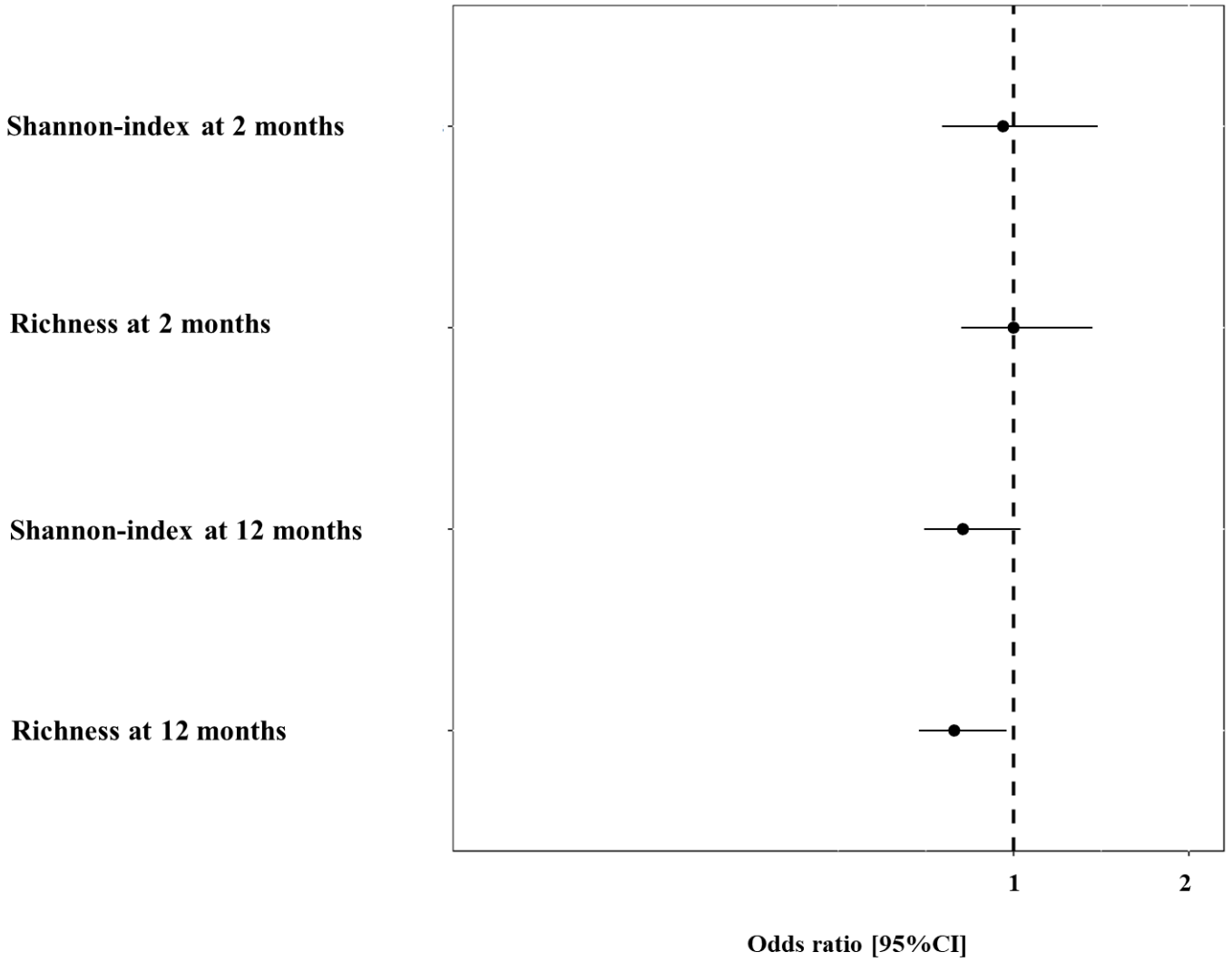


Figure 4.

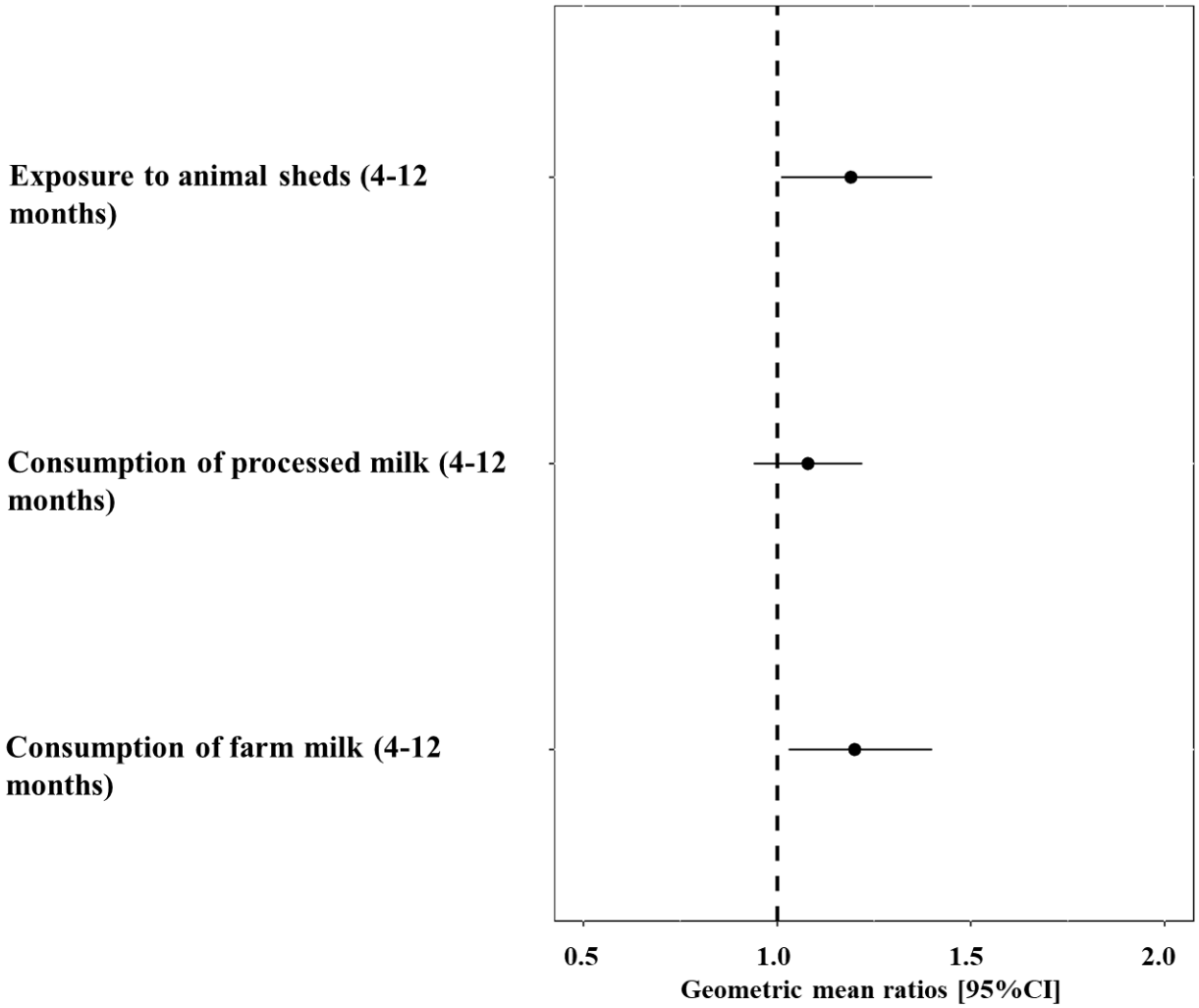
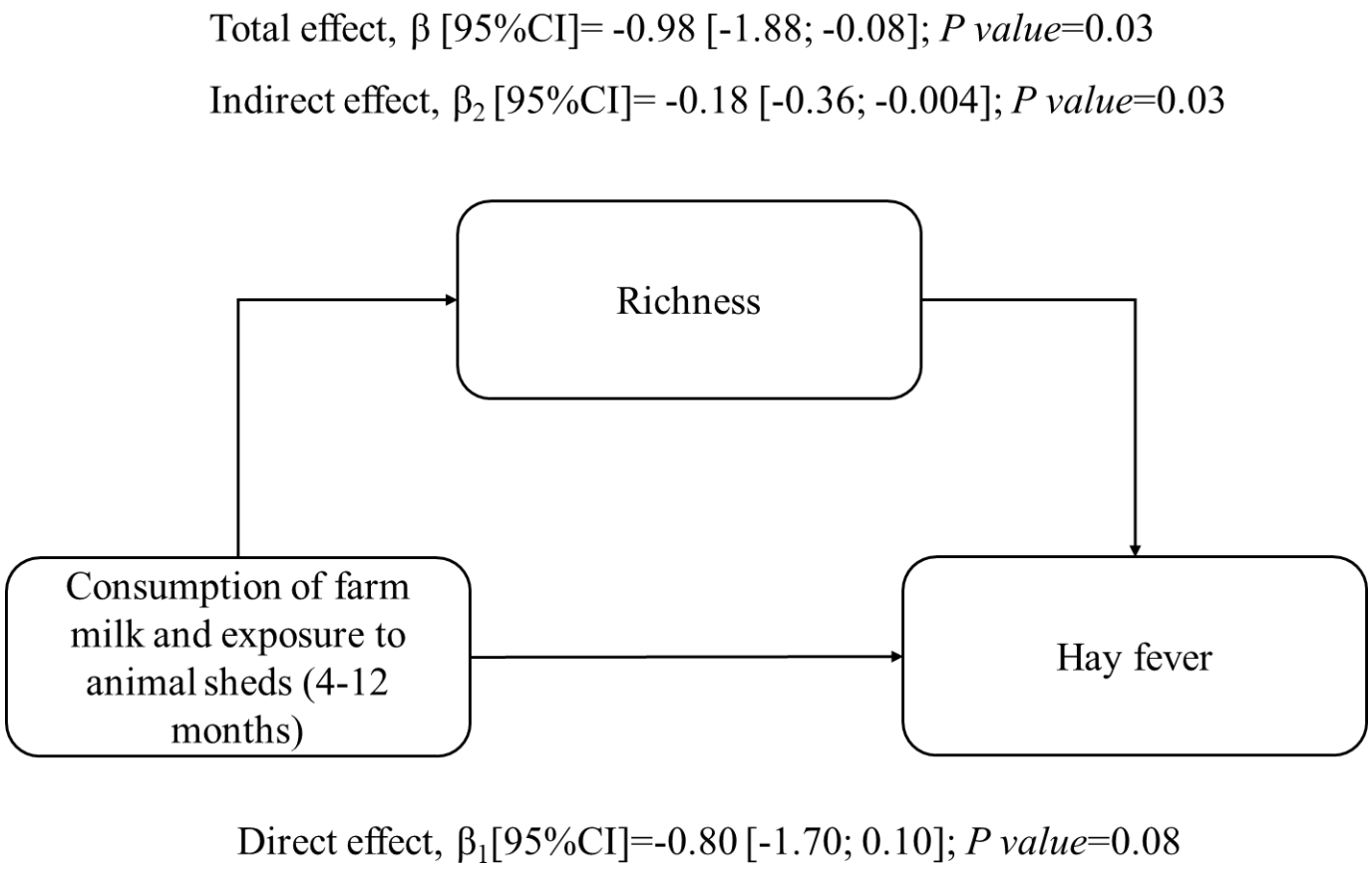

Exposure to animal sheds (4-12 months)
Consumption of processed milk (4-12 months)
Consumption of farm milk (4-12 months)
0.5
1.0
1.5
2.0
Geometric mean ratios [95%CI]

Figure 5.


Total effect, β [95%CI]= -0.98 [-1.88; -0.08]; *P value*=0.03
Indirect effect, β_2 [95%CI]= -0.18 [-0.36; -0.004]; *P value*=0.03
Richness
Consumption of farm milk and exposure to animal sheds (4-12 months)
Hay fever
Direct effect, β_1[95%CI]=-0.80 [-1.70; 0.10]; *P value*=0.08

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Continuous rather than solely early farm exposure protect from hay fever development.

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Methods:

Questionnaires:

Information were collected through mothers using questionnaires in interviews or self-administered questionnaires within the third trimester of pregnancy and when the children were 2, 12, 18 months of age and then at the age of 2, 3, 4, 5, 6, and 10.5 years. Using weekly and monthly diaries and questionnaires from the 8th to 53rd weeks of age, additional information on child's health, nutrition and farm-related exposures were collected (E1, E2).

Definitions of outcome:

Inhalant sensitization at 10.5 years was defined as at least one IgE specific to alder, birch, hazel, plantain, mugwort, alternaria, grass, rye, *Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, cat, dog, or horse at levels $\geq 0.7 IUml^{-1}$ or SPT (birch, grass, alternaria, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, or dog) ≥3mm. Serum specific IgE and SPT was not measured in the Austrian study center. Serum specific IgE was assessed using the semiquantitative Allergy Screen test panel for atopy (Mediwiss Analytic, Moers; Germany) (E3). As described before, SPTs were performed on the anterior part of the forearm using a Stallerpoint ® (Stallergenes, Antony, France) (E4). Incident hay fever at 10.5 years (N=48) was defined by parent reported symptoms (itchy, runny, or blocked nose without a cold accompanied by red itchy eyes) and/or a physician's diagnosis of hay fever in the last 12 months using

questionnaires at age 10.5 years and excluding those having hay fever before the age of 10.5 years.

Assessment of exposures:

Socioeconomic and lifestyle factors, farm-related exposures, health status of women, their husbands and their children were assessed through questionnaires in interviews or self-administered questionnaires to the mothers within the third trimester of pregnancy and when the children were 2, 12, 18 months of age and then at the age of 2, 3, 4, 5, 6, and 10.5 years. Maternal smoking during pregnancy was defined using the following questions "Have you in your life smoked more than 5 packs of cigarettes?" Or "Have you quit smoking in the meantime?" and if yes "Was it during this pregnancy?". Smoking by father, "Have you in your life smoked more than 5 packs of cigarettes?" Or "Do you still smoke?". Second hand smoking was defined by asking "How many cigarettes are on average per day were smoked in your house by other people?" If greater than one then second hand smoking was defined as 1 else 0. Parental education was defined as low (less than 10 years), medium (10 years) and high (greater than 10 years). Parental atopy (yes or no) was defined as doctor's diagnosis of hay fever, atopic dermatitis, or asthma ever in mother or father. Use of antibiotics during pregnancy was defined by asking "Have you taken antibiotics since the beginning of pregnancy?" Or "Have you taken any antibiotics during this pregnancy?". Child was defined as premature if the child was born before the completion of 37 weeks of pregnancy. Use of antibiotics by a child during first year of life was defined as "Total number of weeks with antibiotics ingested". Further, breastfeeding at age of 2 months (yes or no) was defined by asking "if you have ever breastfed?", exposure to pets at age of 2 months (cats and dogs) was defined by asking "if you have cats?", "if you have

dogs?" and "if they stay indoors in the house?", and data on having siblings (yes or no) were also collected. Further, asthma was defined as a physician's diagnosis of asthma or recurrent obstructive bronchitis established until 10.5 years. Eczema and food allergy were defined as physician diagnoses at least once until the age of 10.5 years.

Additionally, the ~~amount~~ frequency of farm milk consumption at each time point by a child (daily, 1-6 times a week, less than once a week or no consumption) was further weighted as follows: weight of 3 was assigned for daily consumption, a weight of 2 for 1-6 times a week, a weight of 1 for consumption less than once a week and 0 for no consumption). The weights over the years were then summed up as farm milk consumption score representing the ~~amount~~ frequency of farm milk consumed. Since data on ~~amount~~ frequency of processed milk consumption was available only at age 10.5 years, instead of constructing a score it was categorized as daily, 1-6 times a week and no (less than once a week or no) consumption of processed milk.

DNA extraction from fecal samples and sequencing analyses:

Briefly, the fecal samples were frozen within 10 minutes of collection, and stored at -20°C until further processing. Targeted DNA amplifications using primers targeting the V4 region of the 16S rRNA gene were performed. The amplicon sequencing was done on Illumina MiSeq instrument producing 250-bp paired end sequences as described previously (E5). Sequencing processing was done using QIIME2-2018.6 (Quantitative Insights Into Microbial Ecology) and reads were denoised using DADA2 (E6, E7). Samples were rarefied at the minimum sequence numbers 1,029. Rarefaction and calculation of richness and Shannon-index was iterated 1,000 times and the resulting measures of α-diversity were then averaged (E5). As described previously, SCFA levels were modeled by the relative abundance of bacterial genera in children

with available SCFA measurements using the "predict" function of R-package ranger (E5). These prediction models were then applied to predict SCFA production scores (butyrate and propionate) in the entire population.

Statistical analyses:

Repeated measure ~~Latent~~ latent classes (exposure classes)

Using farm milk consumption and processed milk consumption exposures together, 3 types of farm and processed milk exposure classes were identified: i) 'high farm and low processed milk', ii) 'mixed consumption of farm and processed milk', and iii) 'low farm and high processed milk' (Figure E2(a)). The children were allocated to specific exposure classes by their highest posterior probabilities. The optimal number of exposure classes was then determined according to the Bayesian Information Criterion. Further, the labelling of the exposure classes was based on main features of each class. The analyses were done on children having data at least at 7 of the 8 assessed time points for the combined farm and processed milk consumption.

The associations between hay fever and farm and shop milk consumption exposure classes was assessed by logistic regression. The above model was adjusted for centers and ~~potential~~ confounders, (growing up on a farm, and parental asthma and/or atopy). We tested the differences in relative abundance of most common single bacterial genera at 2 and 12 months with hay fever by Wilcoxon test, main associations ($p<0.05$) were then confirmed in logistic regression models using center-log-ratio-transformed variables. Gut microbiome richness and Shannon-index at 2 and 12 months were transformed by dividing the original variable by their respective interquartile range (IQR: IQRrichness_2m: 8.07, IQRShannon-index_2m: 0.75, IQRrichness_12m: 15.9 and IQRShannon-index_12m: 0.75) and the new variables were then

included in the regression models (logistic regression to test the association with outcome hay fever at 10.5 years and linear regression to test the associations between consumption of farm milk, consumption of processed milk and exposure to animal sheds in infancy). The association with hay fever is then represented as adjusted odds ratio per IQR of the probability.

E1. Loss G, Depner M, Ulfman LH, van Neerven RJ, Hose AJ, Genuneit J, et al. Consumption of unprocessed cow's milk protects infants from common respiratory infections. J Allergy Clin Immunol. 2015;135(1):56-62.

E2. Loss GJ, Depner M, Hose AJ, Genuneit J, Karvonen AM, Hyvarinen A, et al. The Early Development of Wheeze. Environmental Determinants and Genetic Susceptibility at 17q21. Am J Respir Crit Care Med. 2016;193(8):889-97.

E3. Depner M, Ege MJ, Genuneit J, Pekkanen J, Roponen M, Hirvonen MR, et al. Atopic sensitization in the first year of life. J Allergy Clin Immunol. 2013;131(3):781-8.

E4. Chauveau A, Dalphin ML, Kaulek V, Roduit C, Pugin A, von Mutius E, et al. Disagreement between Skin Prick Tests and Specific IgE in Early Childhood. Int Arch Allergy Immunol. 2016;170(2):69-74.

E5. Depner M, Taft DH, Kirjavainen PV, Kalanetra KM, Karvonen AM, Peschel S, et al. Maturation of the gut microbiome during the first year of life contributes to the protective farm effect on childhood asthma. Nat Med. 2020;26(11):1766-75.

E6. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335-6.

E7. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3.

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Figure E1. Proportion of farm milk consumption and exposure to animal sheds over time in the PASTURE children with data on hay fever at 10.5 years (N=769).

Figure E2. Association of farm milk consumption exposure classes with the stringent definition of hay fever

Models are adjusted for centers, growing up on a farm, and parental atopy. The forest plot represent the aOR with 95%confidence intervals [95%CI].

Figure E3. Farm and processed milk consumption exposure classes

a) Solution for repeated measure latent classes defined by farm and processed milk consumption in the PASTURE children. Numbers in parentheses indicate the total number of children in each class. b) Association of farm and processed milk consumption exposure classes with hay fever. Models are adjusted for centers, growing up on a farm, and parental atopy. The forest plot represent the aOR with 95%CI.

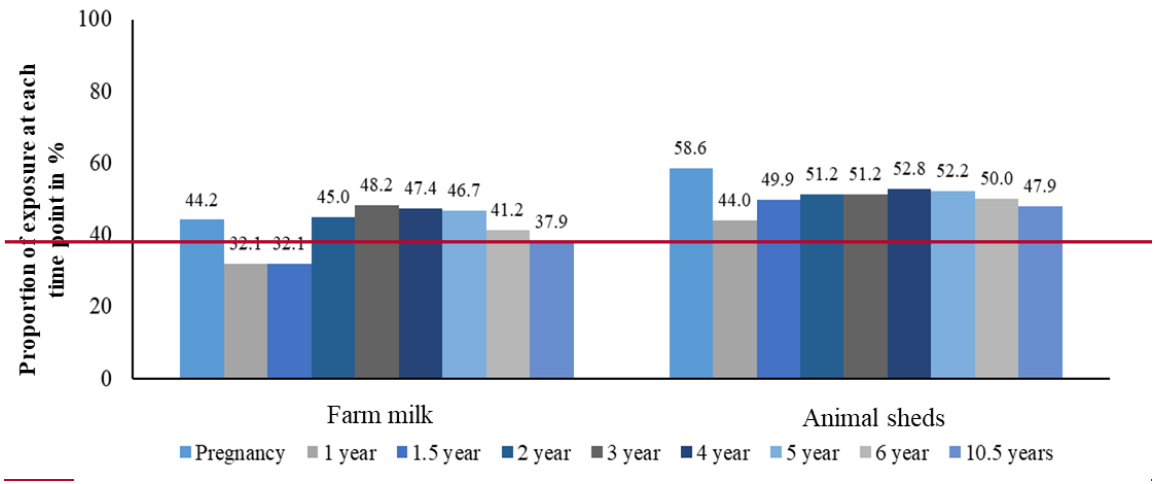
Figure E4. Association of the ~~amount~~ frequency of processed milk consumption at the age of 10.5 years with hay fever at 10.5 years in the PASTURE children.

Model is adjusted for centers, growing up on a farm, and parental atopy. The forest plot represent the aOR with 95%CI.

~~**Figure E5.** Association of the short chain fatty acid butyrate and propionate scores at 12 months with hay fever at 10.5 years.~~

~~Model is adjusted for centers, growing up on a farm, and parental atopy (hay fever (%)/Total: 79 (12.5)/633). The forest plot represent the aOR with 95%CI.~~

~~Figure E1.~~



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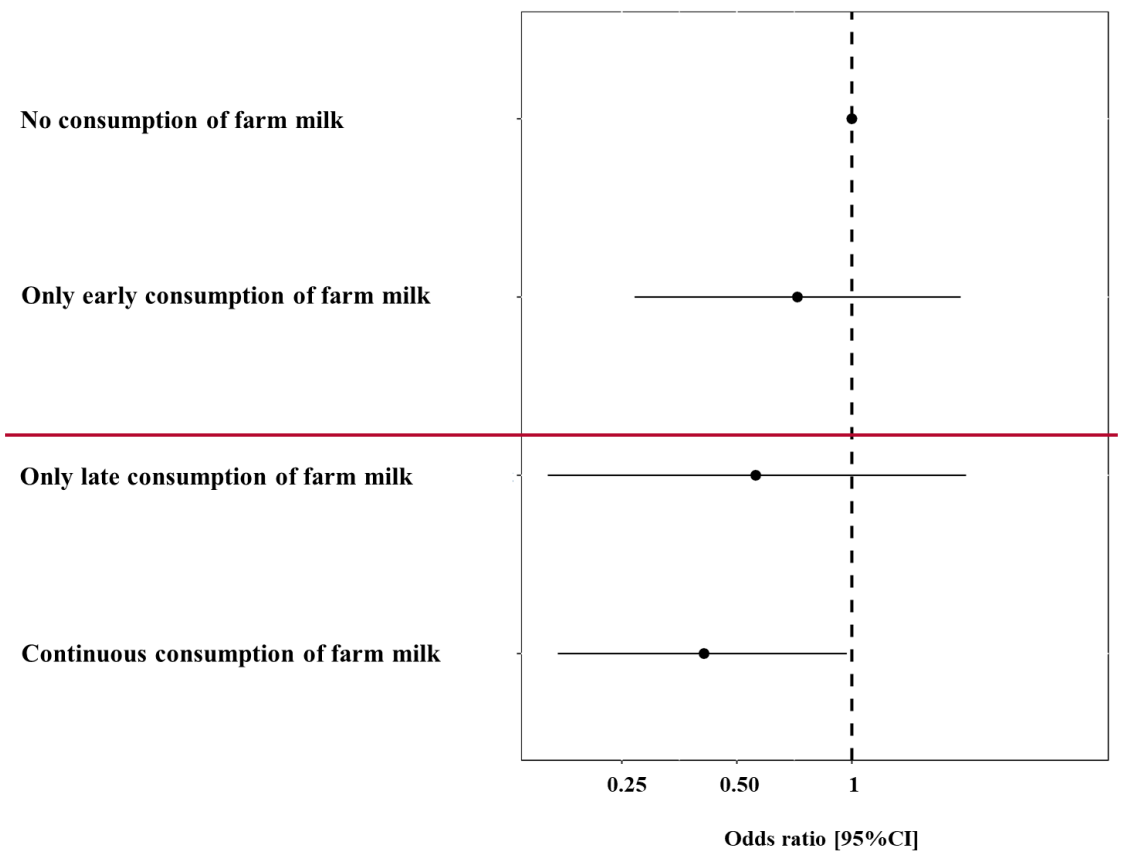
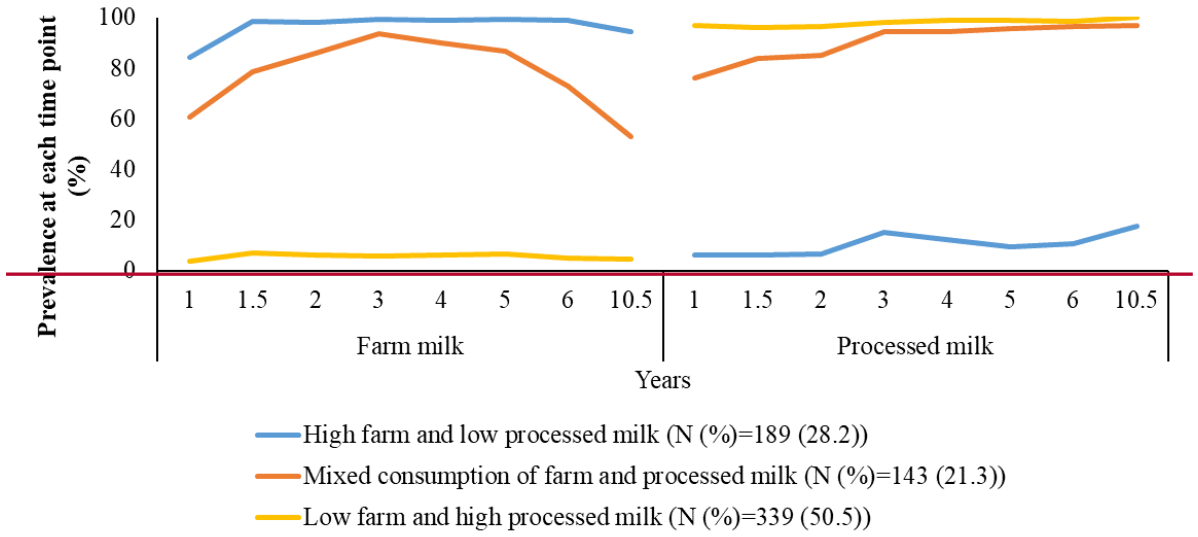
~~Figure E2~~

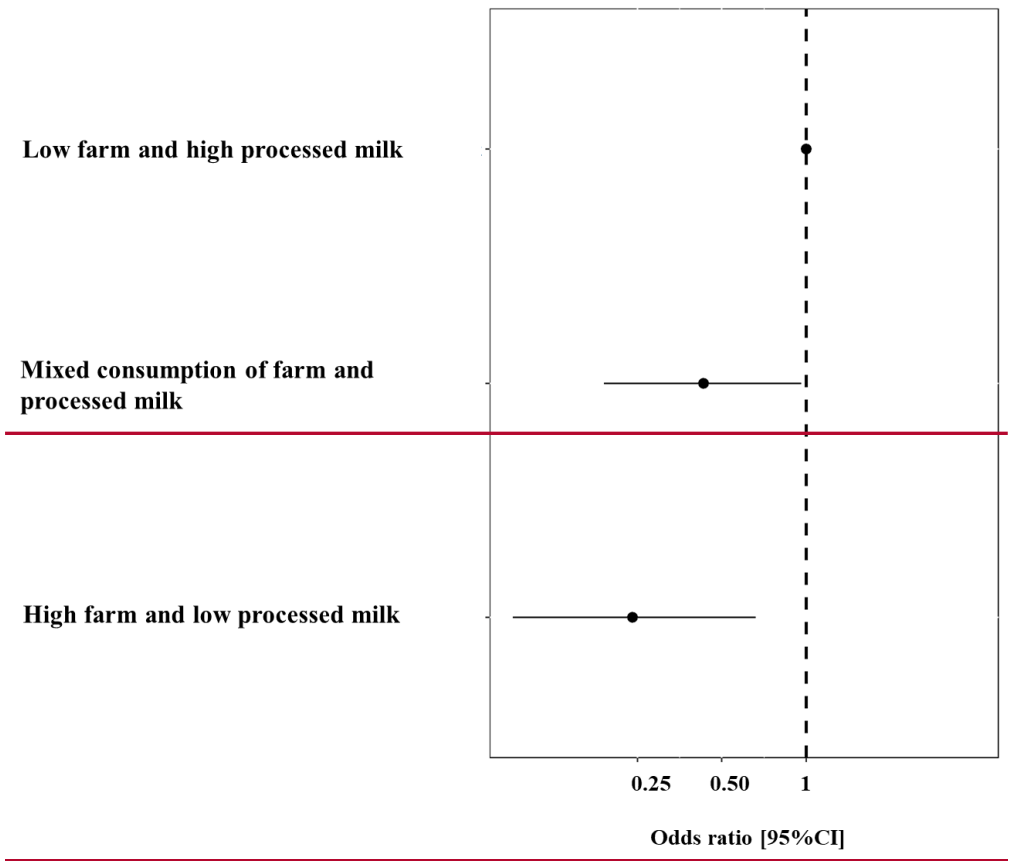

No consumption of farm milk
Only early consumption of farm milk
Only late consumption of farm milk
Continuous consumption of farm milk
0.25
0.50
1
Odds ratio [95%CI]

~~Figure E3.~~

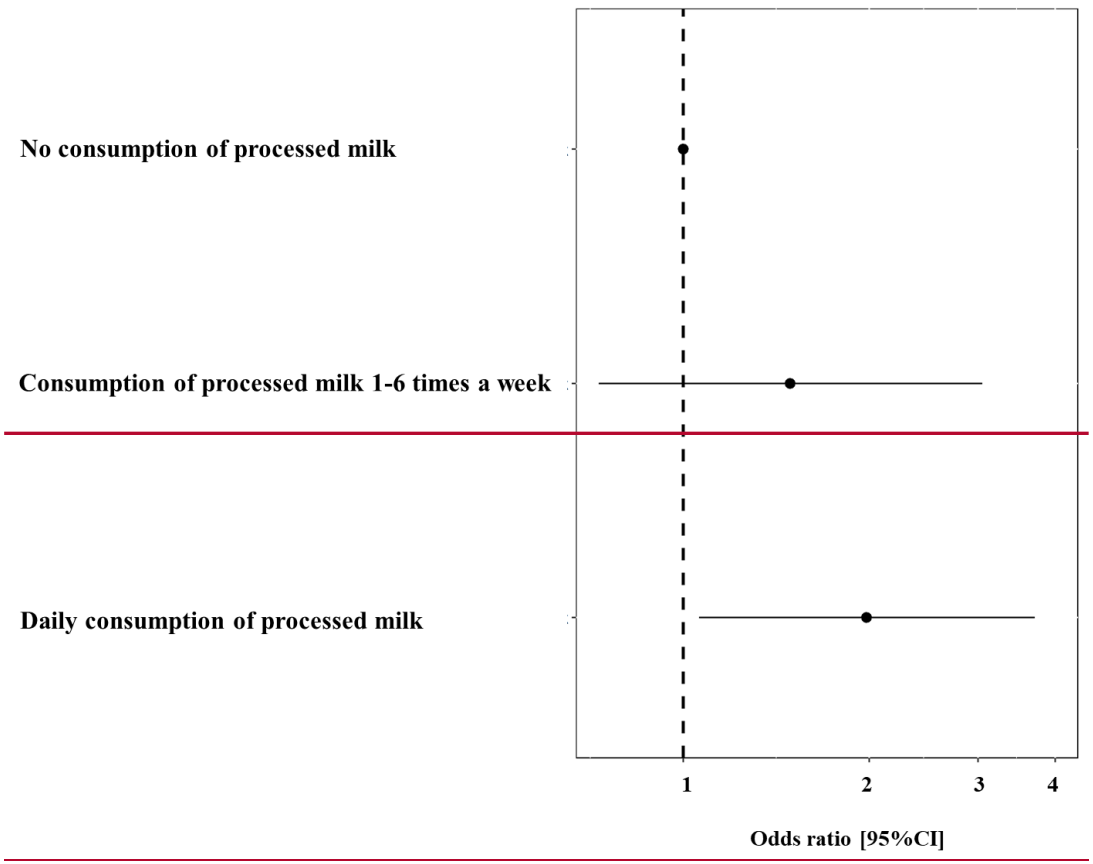
~~(a)~~


Prevalence at each time point (%)
100
80
60
40
20
0
1
1.5
2
3
4
5
6
10.5
1
1.5
2
3
4
5
6
10.5
Farm milk
Processed milk
Years
High farm and low processed milk (N (%)=189 (28.2))
Mixed consumption of farm and processed milk (N (%)=143 (21.3))
Low farm and high processed milk (N (%)=339 (50.5))

~~(b)~~

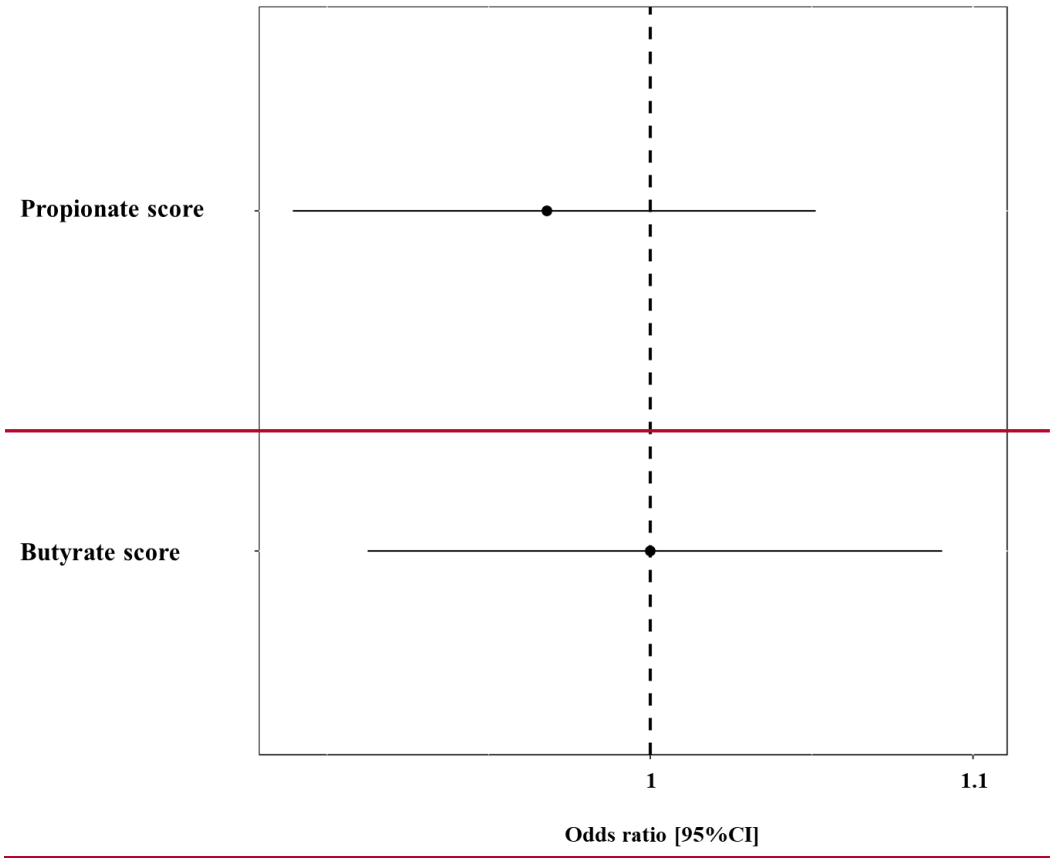

Low farm and high processed milk
Mixed consumption of farm and processed milk
High farm and low processed milk
0.25
0.50
1
Odds ratio [95%CI]

205 **~~Figure E4.~~**



206

207 **~~Figure E5.~~**



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209 **Table E1.** Description of the included and ~~not in~~ex~~c~~luded study population

Characteristic	Included in the study (N=769)	Excluded in the study (N=~~193~~364)	*P value*
	N (%)/Total	N (%)/Total	
Farm child (yes)	367 (47.7)/769	~~98~~ 164 (~~50.8~~45.0)/364	*0.4~~7~~1*
Exposure to cats at age of 2 months (yes)	199 (26.0)/767	~~5~~82 (~~26~~25.9)/323	*0.~~78~~88*
Exposure to dogs at age of 2 months (yes)	147 (19.2)/766	~~21~~48 (1~~4~~0.9)/322	*0.10~~08~~*
Maternal age at pregnancy (years) †	31.3±4.5 (N=769)	30.2±5.0 (N=364)	*<0.003*
Maternal smoking (yes)	96 (12.5)/766	62 (17.0)/363	*0.04*
Second hand smoking (yes)	33 (4.3)/764	16 (5.0)/322	*0.63*
Parental education (yes) Low Medium High	 62 (8.1)/764 280 (36.7)/764 422 (55.2)/764	 63 (18.1)/349 146 (41.8)/349 140 (40.1)/349	 *<0.001*
Use of antibiotics during pregnancy (yes)	204 (27.0)/755	85 (26.1)/326	*0.77*
Parental atopy (yes)	416 (54.4)/765	~~92~~ 176 (~~47~~52.7)/334	*0.~~11~~60*

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Mode of delivery (normal)	624 (81.9)/762	~~155~~ 267 (~~81~~83.~~24~~)/320	*0.~~83~~66*
Premature birth (yes)	11 (1.4)/769	50 (13.7)/364	*<0.0001*
Birth weight (kg) †	3.4±0.4 (N=605)	3.4±0.44 (N=239)	*0.31*
Breast feeding 2 months (yes)	711 (92.7)/767	~~175~~ 294 (~~90~~91.~~70~~)/323	*0.~~36~~27*
Gender (female)	366 (47.7)/768	~~101~~ 166 (~~52~~50.~~39~~)/326	*0.~~26~~36*
Having siblings (yes)	494 (64.2)/769	~~119~~ 227 (~~61~~62.~~4~~3)/364	*0.5~~1~~0*
Use of antibiotics during first year of life (weeks) †	0.03±0.3 (N=746)	0.02±0.1 (N=286)	*0.50*

The categorical variables are presented as frequency (percentage) and the continuous variables as †: me~~di~~an±standard deviation ~~(quartile 1; quartile 3)~~. The test for differences between the groups are $\chi 2$ test for categorical variables and Mann Whitney U test for continuous variables. Farm child was defined as "Children of mothers living on family-run livestock farms were assigned to the farm group. The non-farm group included children of mothers from the same rural areas but not living on a farm". ~~Parental atopy was defined as doctor's diagnosis of hay fever, atopic dermatitis, or asthma ever in mother or father.~~ Exposure to pets at the age of 2 months (cats and dogs) was defined by asking "if you have cats?", "if you have dogs?" and "if they stay indoors in the house?". Maternal smoking during pregnancy was defined using the following questions "Have you in your life smoked more than 5 packs of cigarettes?" Or "Have you quit smoking in the meantime?" and if yes "Was it during this pregnancy?". Smoking by father, "Have you in your life smoked more than 5 packs of cigarettes?" Or "Do you still smoke?". Second hand smoking "How many cigarettes are on average per day were smoked in your house by other people?" If greater than 1 then second hand smoking was defined as 1 else 0. Parental education was defined as low (less than 10 years), medium (10 years) and high (greater than 10 years). Parental atopy was defined as doctor's diagnosis of hay fever, atopic dermatitis, or asthma ever in mother or father. Use of antibiotics during pregnancy was defined by asking "Have you taken antibiotics since the beginning of pregnancy?" Or "Have you taken any antibiotics during this pregnancy?". Child was defined as premature if the child was born before the completion of 37 weeks of pregnancy. Use of antibiotics by a child during first year of life was defined as "Total No. of weeks with antibiotics ingested". Breastfeeding at the age of 2 months (yes or no) was defined by asking "if you have ever breastfed?". ~~Parental atopy was defined as doctor's~~

~~diagnosis of hay fever, atopic dermatitis, or asthma ever in mother or father.~~ Asthma was defined as a physician's diagnosis of asthma or recurrent obstructive bronchitis established until 10.5 years. Eczema and food allergy were defined as physician diagnoses at least once until the age of 10.5 years. NA: not applicable.

226 **Table E2**. Association between animal sheds exposure classes with hay fever at 10.5 years.

	Hay fever/Total	OR [95% CI], *P value*
Model 1		
No exposure to animal sheds	56 (18.0)/312	1
Only early exposure to animal sheds	2 (3.9)/52	0.26 [0.06; 1.15], *0.08*
Only late exposure to animal sheds	9 (12.5)/72	0.88 [0.40; 1.96], *0.76*
Continuous exposure to animal sheds	31 (9.4)/329	1.14 [0.50; 2.64], *0.75*
Model 2		
No exposure to animal sheds	56 (18.0)/312	1
Only early exposure to animal sheds	2 (3.9)/52	0.32 [0.07; 1.44], *0.14*
Only late exposure to animal sheds	9 (12.5)/72	1.04 [0.46; 2.36], *0.92*
Continuous exposure to animal sheds	31 (9.4)/329	1.94 [0.79; 4.74], *0.15*

227 Model 1: adjusted for centers, growing up on a farm, and parental atopy. Model 2: adjusted for centers, farm milk consumption exposure classes, and parental
228 atopy. The number of children included in the analyses are different to that shown in Figure 1(a) due to the missing values of hay fever at year 10.5 years.

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232 **Table E3**. Proportion of children in each exposure classes.

Exposure classes	Hay fever (%)/Total
Farm milk	
No consumption of farm milk	70 (18.9)/371
Only early consumption of farm milk	7 (10.9)/64
Only late consumption of farm milk	6 (11.8)/51
Continuous consumption of farm milk	15 (5.4)/279
Farm milk and processed milk	
Low farm milk and high processed milk	49 (16.2)/302
Mixed consumption of farm milk and processed milk	9 (7.1)/127
High farm milk and low processed milk	7 (4.3)/162

233 Numbers in parentheses indicate percent of children with hay fever in each exposure class. The number of children included in the analyses are different to that
234 shown in Figure 1(b) and Figure E3(a) due to the missing values of hay fever at year 10.5 years.

Table E4. Association between the farm milk exposure classes in different strata with hay fever at 10.5 years.

	Hay fever (%)/Total	OR [95% CI], *P value*
Center Austria *		
— No consumption of farm milk	6 (11.3)/53	1
— Only early consumption of farm milk	0 (0)/8	NA
— Only late consumption of farm milk	1 (9.1)/11	0.78 [0.09; 7.24], *0.83*
— Continuous consumption of farm milk	2 (3.3)/61	0.27 [0.05; 1.38], *0.11*
Center Finland *		
— No consumption of farm milk	25 (26.6)/94	1
— Only early consumption of farm milk	3 (13.0)/23	0.41 [0.11; 1.51], *0.18*
— Only late consumption of farm milk	3 (37.5)/8	1.66 [0.37;7.44], *0.51*
— Continuous consumption of farm milk	2 (5.4)/37	0.16 [0.04;0.70], *0.02*
Center France *		
— No consumption of farm milk	13 (17.3)/75	1
— Early consumption of farm milk	1 (11.1)/9	0.60 [0.07; 5.19], *0.64*
— Late consumption of farm milk	0 (0)/6	NA
— Continuous consumption of farm milk	5 (7.9)/63	0.41 [0.14; 1.23], *0.11*
Center Germany *		
— No consumption of farm milk	15 (18.5)/81	1
— Only early consumption of farm milk	2 (22.2)/9	1.26 [0.24; 6.67], *0.79*

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— Only late consumption of farm milk	2 (12.5)/16	0.63 [0.13; 3.07], *0.56*
— Continuous consumption of farm milk	3 (4.9)/61	0.23 [0.06; 0.83], *0.02*
Center Switzerland *		
— No consumption of farm milk	12 (17.1)/70	1
— Only early consumption of farm milk	1 (6.7)/15	0.35 [0.04; 2.88], *0.33*
— Only late consumption of farm milk	0 (0)/10	NA
— Continuous consumption of farm milk	3 (5.1)/59	0.26 [0.07; 0.97], *0.04*
Excluding children having a family history of parental atopy and avoided milk or milk products **		
— No consumption of farm milk	17 (13.8)/123	1
— Only early consumption of farm milk	1 (3.7)/27	0.25 [0.03; 2.09], *0.20*
— Only late consumption of farm milk	2 (8.3)/24	0.63 [0.13; 3.13], *0.57*
— Continuous consumption of farm milk	5 (3.0)/168	0.21 [0.06; 0.78], *0.02*

*: crude model. Models are adjusted for **: centers, and growing up on a farm.

Table E5. Association of the amount of the farm milk consumption over time with hay fever at 10.5 years

	OR [95% CI], *P value*
Intermediate (28 (10.3)/272) vs Low (60 (19.5)/308)	0.63 [0.37; 1.10], *0.10*
High (10 (5.4)/185) vs Low (60 (19.5)/308)	0.37 [0.16; 0.84], *0.02*

Models are adjusted for centers, growing up on a farm, and parent parental atopy.

Continuous rather than solely early farm exposure protect from hay fever development.

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Source of funding: The PASTURE study was supported by the European Commission (research grants QLK4-CT-2001-00250, FOOD-CT-2006-31708 and KBBE-2007-2-2-06), the European Research Council (grant 250268), Deutsche Forschungsgemeinschaft, German Center for Lung Research (DZL), Kühne Foundation, French national programme for hospital research, EVO- and VTR-funding, the Academy of Finland (grant 139021, 287675, 338679, 339666), the Yrjö Jahnsson foundation, and EU, Academy of Finland and smaller grants from Finnish foundations supporting medical research (Juho Vainio Foundation, Päivikki and Sakari Sohlberg foundation, Finnish cultural foundation). The current analyses were supported by the Bavarian State Ministry for Health and Care.

Conflict of interest:

S.P.: reports support for attending virtual ERS International Congress 2021. C.R.: reports payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from ALK, University of Bern. C.S.: reports grants from Universities Giessen and Marburg Lung Center, German Center for Lung Research, University Hospital Giessen and Marburg research funding, Deutsche Forschungsgemeinschaft (DFG) (SFB1021 (C04), KFO 309 (P10), SK 317/1-1 (Project Nr. 428518790)), TransMIT, Mead Johnson Nutrition; Consulting fees: Hycor Biomedical, Bencard Allergie, Thermo Fisher Scientific; Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events: Hycor Speaker Bureau; Support for attending meetings and/or travel: Hycor, Bencard Allergy; Participation on a Data Safety Monitoring Board or Advisory Board: Hycor

Biomedical, Bencard Allergy; Leadership or fiduciary role: ESGREV, DZL ALLIANCE; Receipt of equipment, materials, drugs, medical writing, gifts or other services: Bencard Allergie. C.B.: reports support for attending meetings/travel from GSK, Sanofi, Chiesi, Novartis, AstraZeneca. M.J.E.: reports support for the present manuscript from German Center for Lung Research (DZL); Grants or contracts from any entity: Dutch Longfonds; Patents planned, issued or pending: EP000001964570B1, US000009950017B2, EP000002361632B1; Receipt of materials: Milk powder from Friesland Campina. A.D-C.: reports support for the present manuscript from French national programme for hospital research; Grants/contracts: French public agency ANSES, Don du Souffle for the PASTURE 16-yr Visit, Fondation du Souffle for the PASTURE 16-yr visit, Novartis, ARAIRLOR (Association Régionale d'Aide aux Insuffisants Respiratoires de Lorraine); Consultant fees: Stallergens, ALK, Aimmune Therapeutics; Payment/Honoria: Aimmune Therapeutics (speaker at symposium at the EAACI congress in 2019 and at the french congress of Allergy in 2019), Novartis; Attending meetings: Mead Johnson for Pediatric Allergy and Asthma Meeting 2019, Nutricia for EAACI congress 2019, Aimmune Therapeutics for EAACI congress 2019, Novartis for the French congress of Allergy 2019; Stock: Essilor Luxottica. B.S. reports support for the present manuscript from DFG, and EU; Grants/contracts: DFG, and EU. R.L.: reports support for the present manuscript from Kühne Foundation, and EU European Commission (research grant QLK4-CT-2001-00250); A.M.K: reports support for the present manuscript from EVO- and VTR-funding, the Academy of Finland (grant 139021, 287675, 338679, 339666), the Juho Vainio Foundation, the Yrjö Jahnsson foundation, Päivikki and Sakari Sohlberg foundation, the Finnish Cultural Foundation. J.P.: reports support for the present

manuscript: EU, Academy of Finland and smaller grants from Finnish foundations supporting medical research (Juho Vainio Foundation, Päivikki and Sakari Sohlberg foundation, Finnish cultural foundation. E.v.M.: reports support for the present manuscript: Funding PASTURE study, FORALLVENT study, EFRAIM study and Leibniz prize; Grants: Bavarian State Ministry of Health and Care for “URS Study”, 01/21, German Federal Ministry of Education and Research (BMBF) *Deutsches Zentrum für Lungenforschung [German Center for Lung Research],* 01/21, Forschungsvorhaben: Rolle der mikrobiellen Umweltexposition für den Schutz vor Heuschnupfen und Asthma Research project: The role of the microbial environmental exposure for the protection against hay fever and asthma *Bayerisches Staatsministerium für Gesundheit und Pflege Bavarian State Ministry of Health and Care,* 01/20; Royalties or licenses: Elsevier GmbH, Georg Thieme Verlag, Springer-Verlag GmbH, Elsevier Ltd., Springer Nature Group; Consulting fees: Chinese University of Hongkong, European Commission, HiPP GmbH & Co KG, AstraZeneca, Imperial College London, OM Pharma, ALK-Abello Arzneimittel GmbH; Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events: Massachusetts Medical Society, Springer-Verlag GmbH, Elsevier Ltd., Böhringer Ingelheim International GmbH, European Respiratory Society (ERS), Universiteit Utrecht, Faculteit Diergeneeskunde, Universität Salzburg, Springer Medizin Verlag GmbH, Japanese Society of Pediatric Allergy and Clinical Immunology (JSPACI), Klinikum Rechts der Isar, University of Colorado, Paul-Martini-Stiftung, Astra Zeneca, Imperial College London, Children´s Hospital Research Institute of Manitoba, Kompetenzzentrum für Ernährung (Kern), OM Pharma S.A., Swedish Pediatric Society for Allergy and Lung Medicine, Chinese

College of Allergy and Asthma (CCAA), ALK-Abello Arzneimittel GmbH, Abbott Laboratories, Deutscher Apotheker Verlag GmbH & Co. KG; Support for attending meetings and/or travel: Verein zur Förderung der Pneumologie am Krankenhaus Großhansdorf e.V., Pneumologie Developpement, Mondial Congress & Events GmbH & Co. KG, American Academy of Allergy, Asthma & Immunology, Imperial College London, Margaux Orange, Volkswagen Stiftung, Böhringer Ingelheim International GmbH, European Respiratory Society (ERS), Universiteit Utrecht, Faculteit Diergeneeskunde, Österreichische Gesellschaft f. Allergologie u. Immunologie, Massachusetts Medical Society, OM Pharma S. A., Hanson Wade Ltd., iKOMM GmbH, DSI Dansk Borneastma Center, American Thoracic Society, HiPP GmbH & Co KG, Universiteit Utrecht, Faculteit Bètawetenschappen; Patents planned, issued or pending: EvM has patent No. PCT/EP2019/085016 (Barn dust extract for the prevention and treatment of diseases) pending (Barn dust extract for the prevention and treatment of diseases) pending, royalties paid to ProtectImmun for patent EP2361632 (Specific environmental bacteria for the protection from and/or the treatment of allergic, chronic inflammatory and/or autoimmune disorders, granted on 19 March 2014), and patents EP1411977 (Composition containing bacterial antigens used for the prophylaxis and the treatment of allergic diseases, granted on 18 April 2007), EP1637147 (Stable dust extract for allergy protection, granted on 10 December 2008), and EP 1964570 (Pharmaceutical compound to protect against allergies and inflammatory diseases, granted on 21 November 2012) licensed to ProtectImmun. Patent EP21189353.2. 2021. von Mutius E, Rankl B, Bracher F, Müller C, Walker A, Hauck SM, Merl-Pham J, inventors; Proteins identified from bran dust extract for the prevention and treatment of

diseases. Patent PCT/US2021/016918. 2021. Martinez FD, Vercelli D, Snyder SA, von Mutius E, Pivniouk V, Marques dos Santos M, inventors; Therapetic fractions and proteins from athma-protective farm dust; Participation on a Data Safety Monitoring Board or Advisory Board: Member of the EXPANSE (funded by European Commission) Scientific Advisory Board, Member of the BEAMS External Scientific Advisory Board (ESAB), Member of the Editorial Board of “The Journal of Allergy and Clinical Immunology: In Practice”, Member of the Scientific Advisory Board of the Children’s Respiratory and Environmental Workgroup (CREW), Member of the International Scientific & Societal Advisory Board (ISSAB) of Utrecht Life Sciences (ULS), University of Utrecht, Member of External Review Panel of the Faculty of Veterinary Science, University of Utrecht, Member of the Selection Committee for the Gottfried Wilhelm Leibniz Programme (DFG), Member of the International Advisory Board of Asthma UK Centre for Applied Research (AUKCAR), Member of the International Advisory Board of “The Lancet Respiratory Medicine”, Member of the Scientific Advisory Board of the CHILD (Canadian Healthy Infant Longitudinal Development) study, McMaster University, Hamilton, Canada, Asthma UK Centre for Applied Research, Pediatric Scientific Advisory Board Iceland. All other authors have no conflict of interest in relation to this work. J.G: reports payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from The journal Pediatric Allergy and Immunology (co-owned by Wiley and EAACI); personal honorarium for serving as Associate Editor. M.K: reports grants or contracts from European Union, German Ministry of Education and Research, German Research Foundation, Infectopharm; consulting fees from Bionorica, Sanofi, Novartis, Bencard; payment or honoraria for

lectures, presentations, speakers bureaus, manuscript writing or educational events from ERS, EAACI, ATS, Novartis, Glaxo, Chiesi, Sanofi, Nutricia, Hipp, Allergopharma; patents planned, issued or pending METHOD FOR TESTING A SUBJECT THOUGHT TO HAVE OR TO BE PREDISPOSED TO ASTHMA European patent application 5 EP07301135.5. Other co-authors declares no conflict of interests.

Abstract word count: 239

Manuscript word count: 3650

Abstract

Background: An important 'window of opportunity' for early life exposures has been proposed for the development of atopic eczema and asthma.

Objective: However it is, unknown whether hay fever with a peak incidence around late school age to adolescence is similarly determined very early in life.

Methods: In the PASTURE birth cohort potentially relevant exposures such as farm milk consumption and exposure to animal sheds were assessed at multiple time points from infancy to age 10.5 years and classified by repeated measure latent class analyses (N=769). Fecal samples at age 2 and 12 months were sequenced by 16S rRNA. Hay fever was defined by parental reported symptoms and/or physician's diagnosis of hay fever in the last 12 months using questionnaires at age 10.5 years.

Results: Farm children had half the risk of hay fever at age 10.5 years (adjusted odds-ratio (aOR) [95% CI]=0.50 [0.31; 0.79]) compared to non-farm children. While early life events such as gut microbiome richness at age 12 months (aOR=0.66 [0.46; 0.96]) and exposure to animal sheds in the first three years of life (aOR=0.26 [0.06; 1.15]) were determinants of hay fever, the continuous consumption of farm milk from infancy up-to school age was necessary to exert the protective effect (aOR=0.35 [0.17; 0.72]).

Conclusion: While early life events determine the risk of subsequent hay fever, continuous exposure is necessary to achieve protection. These findings argue against the notion that only early life exposures set long-lasting trajectories.

Highlight box:

1. What is already known about this topic?

The protective effects of early life farm exposures and gut microbiome composition on atopic diseases and asthma proposes an important window of opportunity.

2. What does this article add to our knowledge?

Early life farm exposures also determine risk of hay fever. However, continuous farm milk consumption is necessary for optimal prevention, thereby arguing against the notion of an early-determined trajectory governing later outcomes.

3. How does this study impact current management guidelines?

These results emphasize the preventive potential of continuously drinking unprocessed farm milk for hay fever protection, suggesting carrying out clinical trials to test microbiologically safe cow's milk for protection from hay fever.

Keywords: Childhood, farm milk, farming, gut microbiome, hay fever, animal sheds.

Abbreviations:

PASTURE: Protection against Allergy-Study in Rural Environments

IgE: immunoglobulin E

SPT: skin prick test

RMLCA: repeated measure latent class analyses

q: quintile

aOR: adjusted odds ratio

95%CI: 95% confidence interval

IQR: interquartile range

Introduction

Hay fever is the most common allergic disease worldwide with a prevalence between 20-30% (1). The high prevalence has a vast impact on several factors such as quality of life and high healthcare costs (2, 3). Numerous epidemiological studies have shown the protective effect of early life farm exposures and gut microbiome composition on asthma, atopy, atopic sensitization, and hay fever (4-11), thus, proposing an important 'window of opportunity' for early life farm exposures and gut microbiome composition for the protection of atopic diseases and asthma. However, it is unknown whether hay fever with a peak incidence around late school age to adolescence is only determined very early in life or whether later exposure before the onset of disease matters most.

The protective "farm-effect" has been attributed to two factors; consumption of unprocessed cow's milk, subsequently termed 'farm milk' and exposure to animal sheds (12-16). Hence, the aim of these analyses is to study the temporal pattern of these protective exposures on hay fever development using the longitudinal data from the PASTURE study. Furthermore, the role of the gut microbiome was investigated.

Methods

Study design and population

PASTURE is a prospective birth cohort study started in 2002 and is conducted in children from rural areas of 5 European countries (Austria, Finland, France, Germany, and Switzerland) (17). The study was designed to evaluate risk and preventive factors for atopic diseases. The study was approved by the local research ethics committees in each country, and written informed consent were obtained from the children's parents. Pregnant women were invited to participate during their third trimester of pregnancy. The children from the participating women were recruited at birth. Children of mothers living on family-run livestock farms at birth of the children were assigned to the farm group. The non-farm group included children of mothers from the same rural areas but not living on a farm (18). Information were obtained through questionnaires in interviews or self-administered questionnaires from mothers.

Definitions of outcome:

Hay fever was defined by parent reported symptoms (itchy, runny, or blocked nose without a cold accompanied by red itchy eyes) and/or a physician's diagnosis of hay fever in the last 12 months using questionnaires at age 10.5 years. Allergen specific IgE and skin prick test (SPT) were assessed at age 10.5 years (19). Inhalant sensitization was defined as at least one IgE specific to alder, birch, hazel, plantain, mugwort, alternaria, grass, rye, *Dermatophagoides pteronyssinus*, *Dermatophagoides farina,* cat, dog, or horse at levels ≥0.7IUml^{-1} or SPT (birch, grass, alternaria, *Dermatophagoides pteronyssinus, Dermatophagoides farinae,* cat, or dog) ≥3mm. A more stringent

267 definition of hay fever consisting of hay fever plus inhalant sensitization at 10.5 years
268 was used in sensitivity analyses.

269 *Assessment of exposures:*

270 The child's consumption of any farm milk, pasteurized and homogenized milk
271 subsequently termed "processed milk" consumption, and any exposure to animal sheds
272 (cows, pigs, sheep, or horses) at time points 12, 18 months, 2, 3, 4, 5, 6, and 10.5 years
273 were assessed. In addition, maternal any farm milk consumption and animal sheds
274 exposure was assessed during pregnancy and infant's consumption of any farm milk,
275 processed milk and exposure to animal sheds (month 4-12) were obtained on weekly
276 basis by diary. The exposure to animal sheds was further dichotomized based on third
277 quartile (17 weeks) weeks spent on animal sheds as a cut-off.

278 Avoidance of milk or milk products was assessed at the age of 12, 18 months, 2, 3, 4, 5,
279 and 6 years. Additionally, information on frequency of farm milk consumption was
280 assessed at the age of 18 months, 2, 3, 4, 5, 6 and 10.5 years of age. Frequency of
281 processed milk consumption was assessed at age 10.5 years.

282 *DNA extraction from fecal samples and sequencing analyses:*

283 Fecal samples were collected from children's diapers during the home visit at the age of
284 2 and 12 month. DNA was extracted from homogenized samples and bioinformatics
285 processing were performed as previously described in detail (10). Briefly, α-diversity
286 (i.e. richness and Shannon-index) was calculated as average of multiple times rarefied
287 samples (10). Metabolite levels of short chain fatty acids (SCFA) were measured in

fecal samples obtained from 301 children at the age of 12 months (20, 21). Two variables, butyrate and propionate scores were created by modeling SCFA-levels on the relative abundance of all bacterial genera using random forest model in the R-package ranger.

Statistical analyses

We performed repeated measure latent class analyses (RMLCA) using data from pregnancy to age 10.5 years i.e. 9 time points were included separately for exposure to animal sheds, and farm milk consumption (Figure 1(a-b)). The children were allocated to specific exposure classes by their highest posterior probabilities. The analyses were done on children having data at least at 7 of the 9 assessed time points. The optimal number of exposure classes was then determined according to the Bayesian Information Criterion and the labelling of the exposure classes was based on main features of each class.

Further as sensitivity analyses, we repeated the farm milk RMLCA, in subgroup of children without a family history of parental asthma and/or atopy and excluding children avoiding milk or milk products at the age 1–6 years as it could introduce confounding by reverse causation, i.e. a positive family history. A farm milk consumption score (Methods section in the Online Repository Text) reflecting the frequency of farm milk consumed was built and divided into quintiles. The quintiles were further categorized as low (q1), intermediate (q2-q4) and high (q5).

The associations between hay fever and potential exposures (farm milk exposure classes, animal sheds exposure classes, frequency of farm milk consumption

(continuous and quintiles), frequency of processed milk consumption, SCFAs (butyrate score and propionate score) as well as gut microbiome's richness, and Shannon-index) were assessed by logistic regression. We tested the differences in relative abundance of most common single bacterial genera at 2 and 12 months with hay fever by Wilcoxon test (10). The associations between gut microbiome richness and farm milk consumption, processed milk consumption and exposure to animal sheds during infancy was assessed by linear regression. The effect estimates are presented as adjusted odds ratios (aORs) for logistic regression and geometric mean ratios (GMR; calculated by exponentiation of the regression coefficients and their 95% confidence intervals (95%CI)) for linear regression along with their respective 95%CI and a *P value* of 0.05 was considered significant. The above models were adjusted for centers and confounders (growing up on a farm and parental asthma and/or atopy) associated with hay fever and exposures in our study. No other confounders i.e. associated with both outcome and exposures were found. We additionally calculated the Number Needed to Treat (NNT), which is the effectiveness of a treatment on an outcome using an R-script (22).

Furthermore, we conducted mediation analyses to assess whether the associations between farm milk consumption and exposure to animal sheds in infancy (4-12 months) and the risk of hay fever is mediated by gut microbiome features adjusting for centers. The mediation analysis was conducted through path analysis using maximum likelihood test to estimate the regression parameters in Mplus 8.5 (23). The mediating effect is reported as the proportion of the estimated indirect effect to the total effect.

The statistical analyses were performed with SAS 9.4 software (SAS Institute, Cary, NC) and Mplus 8.5 software (Muthén & Muthén, Los Angeles, California).

Results

Characteristics of the study population

At 10.5 year follow up 778 children participated in the PASTURE study and 769 have data on hay fever. Comparing the baseline characteristics between included (N=769) and excluded children (N=364) did not show any significant difference except for maternal age at pregnancy, maternal smoking, parental education, and premature birth (Table E1 Online Repository Text). Data on farm milk consumption and exposure to animal sheds at least at one time point (from pregnancy, age of 12, 18 months, 2, 3, 4, 5, 6, and 10.5 years) was available for all these children. Of these, 769 children had information on hay fever at 10.5 years of age. The proportion of children growing up on a farm was 47.7%. Hay fever at the age of 10.5 years was reported in 12.9% children. Of these, 28.9%, 36.7%, and 21.7% had asthma, eczema, and food allergy at age 10.5 years respectively (Table 1). Further, 86.8% were sensitized to inhalant allergens at age 10.5 years (Table 1). Figure E1 (Online Repository Text) shows the proportion of children who were consuming farm milk or were exposed to animal sheds at each time point. The consumption of farm milk by children increased from the age of 1 to 3 years and gradually decreased after age 4 years. Similarly, exposure to animal sheds also increased from the age of 1 to 4 years and slightly decreased after age 5 years.

Temporal pattern of the farm-related exposures on hay fever

Children growing up on a farm had half the risk of hay fever as compared to non-farm children (aOR [95%CI], *P value*: 0.50 [0.31; 0.79], *0.003*).

In a first step, we analyzed the temporal pattern of exposure to animal sheds (‘continuous exposure to animal sheds’, ‘only early exposure to animal sheds’, ‘only late exposure to animal sheds’ and ‘no exposure to animal sheds’; Figure 1(a)) on hay fever development. Of these categories, ‘only early exposure to animal sheds’ showed an inverse association when compared to ‘no exposure to animal sheds’ which however did not reach statistical significance (0.26 [0.06; 1.15], *0.08*) (Table E2 Online Repository Text). When adjusting this model for consumption of farm milk exposure classes, the results remained unchanged (Table E2 Online Repository Text).

We then analyzed the temporal pattern of consumption of farm milk in similar categories ‘continuous consumption of farm milk’, ‘only early consumption of farm milk’, ‘only late consumption of farm milk’ and ‘no consumption of farm milk’ (Figure 1(b)). The strongest inverse association was found for the ‘continuous consumption of farm milk’ as compared to ‘no consumption of farm milk’ (0.35 [0.17; 0.72], *0.004*) exposure class (Figure 2 and Table E3 Online Repository Text). In contrast, ‘only early consumption of farm milk’ showed no significant effect on hay fever. The inverse association of ‘continuous consumption of farm milk’ compared to ‘no consumption of farm milk’ was still observed when using the stringent definition of hay fever (0.41 [0.17; 0.97], *0.04*) (Figure E2 Online Repository Text) or incident hay fever at age 10.5 years (0.39 [0.15; 0.99], *0.05,* data not shown). Since confounding by reverse causation might have biased our findings, we ran a sensitivity analysis in the subgroup of children without a family history of parental asthma and/or atopy and excluded children avoiding milk or milk products at the age 1–6 years. This did not change the inverse association with hay fever (0.21 [0.06; 0.78], *0.02*, data not shown).

We next assessed the association of the frequency of farm milk consumption i.e. whether frequently drinking farm milk has a dose-response effect on hay fever. The highest compared to the lowest quintile of farm milk consumption was inversely associated with hay fever (0.37 [0.16; 0.84], *0.02*), whereas the intermediate group (q2-q4; 0.63 [0.37; 1.10], 0.10) showed a similarly inverse but non-significant association. Similar results were obtained when using frequency of farm milk consumption score as a continuous variable (data not shown).

We further investigated if consumption of processed milk shows similar effects as consumption of farm milk (Figure E3(a) Online Repository Text). Consumption of 'high farm and low processed milk' was inversely associated with hay fever (0.24 [0.09; 0.66], *0.006)*, however, the consumption of processed milk attenuated the farm milk effect when both farm milk and processed milk were consumed ('mixed consumption of farm and processed milk' (0.43 [0.19; 0.96], *0.04)* (Figure E3(b) and Table E3 Online Repository Text). Furthermore, daily consumption of shop milk at the age of 10.5 years showed association in positive direction with hay fever (Figure E4 Online Repository Text).

Additionally, NNT calculated in our study was 7.14, i.e. 7 children would have to drink farm milk continuously from pregnancy by mothers until age 10.5 years in order to prevent hay fever in one child.

Early life effect of gut microbiome on hay fever

We investigated the role of the early life gut microbiome by relating bacterial composition, richness, Shannon-index (at age 2 and 12 months) and SCFA to hay fever.

We did not find any significant differences in relative abundance of most common bacterial genera at 2 and 12 months with subsequent hay fever at 10.5 year (data not shown). Also, richness and Shannon-index of bacteria at 2 months were not associated with hay fever at 10.5 years (Figure 3). However, the bacterial richness of the gut microbiome at 12 months was inversely associated with hay fever (aOR [95%CI], *P value*: 0.66 [0.46; 0.96], *0.03,* Figure 3). Shannon-index at 12 months also showed an inverse non-significant trend for hay fever (0.71 [0.49; 1.04], *0.08,* Figure 3). The SCFAs butyrate (1.00 [0.92; 1.09], *0.99*) and propionate scores (0.97 [0.90; 1.05], *0.50*) were in turn not associated with hay fever (data not shown). We reasoned that consumption of milk and exposure to animal sheds may shape the gut microbiome, in particular its richness. Consumption of farm milk (aGMR [95%CI]: 1.20 [1.03; 1.40], *P value=0.02*) and exposure to animal sheds (aGMR [95%CI]: 1.19 [1.01; 1.40], *P value=0.04*) in the first year of life increased gut microbiome richness (Figure 4). In turn, no association was observed for consumption of processed milk (Figure 4). Since both, farm milk consumption and exposure to animal sheds during infancy (4-12 months) showed significant associations with gut microbiome richness at 12 months, we performed a mediation analysis including unexposed and children exposed to both in infancy. The mediation analysis revealed that part (18.4%) of the total protective effect of farm milk consumption and exposure to animal sheds in the first year of life on hay fever was mediated by gut microbiome richness (*P value=0.03*, Figure 5). The number of children

421 only being exposed to animal sheds or farm milk, respectively, was too low to allow
422 separate mediation analyses.

Discussion

In the PASTURE birth cohort, the continuous consumption of farm milk throughout age 10.5 years, but neither the only early nor the only late exposure alone was significantly associated with reduced risk of hay fever at age 10.5 years. In contrast, exposure to animal sheds only exerted a trend towards protection early in life. Both exposures, farm milk and animal sheds, early in life increased gut microbiome richness at age 12 months, which partly explained the protective effect of these exposures on hay fever.

The human gut microbiome composition plays an important role in shaping the development of the immune system (24). There is some evidence that the gut microbiome diversity in the first years of life may protect from atopic sensitization. In the population based CHILD cohort, the Shannon-index at age 3 months was associated with protection from atopic sensitization at 1 year (8). However, in a Swedish study the Shannon-index in early infancy was not associated with allergic rhinoconjunctivitis and SPT at age 7 years (25). Our analyses likewise do not confirm this very early ‘window of opportunity’ since gut microbiome richness and Shannon-index at age 2 month was unrelated to hay fever development.

In contrast, gut microbiome richness at the age of 1 year was inversely associated with hay fever at age 10.5 years. We have previously shown in the PASTURE cohort in agreement with others that the compositional structure of the gut microbiome undergoes very significant changes from early age when most infants are breastfed to age 12 months when most foods have been introduced into a child’s diet (10, 11). Nevertheless, an inverse association of gut microbiome richness at age 1 year with an

445 outcome much later in life at age 10.5 years may seem surprising. This long-term
446 association may be attributable to an earlier onset of disease. In fact, 4.6%, 5.9% and
447 6.7% of children with data on hay fever at age 10.5 years had already reported
448 symptoms and/or a diagnosis of hay fever at age 4, 5 and 6 years, respectively.
449 Furthermore, early alterations of the composition of the gut microbiome may shape its
450 subsequent development towards an adult-like compositional structure in the first 3
451 years of life (26). Unfortunately, no fecal samples have been collected at later time
452 points in the PASTURE cohort.
453 The production of the SCFAs butyrate and propionate measured at 12 months of age
454 has been reported previously as determinants of protection against atopic sensitization
455 at age 6 years (20). In our study, no relation between the SCFAs butyrate and
456 propionate with hay fever was found. Furthermore, no association with single taxa was
457 seen. Thus, different facets of the early development of the gut microbiome composition
458 may matter for different clinical outcomes.
459 Of the environmental exposures investigated in these analyses, the continuous, but
460 neither the early nor the late, consumption of farm milk was seen to protect from hay
461 fever development. Moreover, a dose-response effect was found corroborating the
462 strength of the observation. Interestingly, this protective effect was partly mediated by
463 gut microbiome richness which may suggest that a continued exposure to unprocessed
464 cow’s milk may increase gut microbiome richness beyond the age of 12 months and
465 thereby confer its protective effect.

Continuous exposure also implies repeated exposures. The novel concept of trained immunity may lend itself to mechanistic speculations since phenomena like LPS tolerance are based on the necessity of repeated rather than single exposures (27).
A potential explanation for the differential effect of unprocessed versus processed cow's milk is grounded in the observation that most farm children drink their milk unboiled. In fact, too few children received only boiled, i.e. heat treated farm milk over the study period to allow meaningful stratified analyses. A number of population-based and experimental studies have stressed the potential importance of heat-treatment of cow's milk for the loss of protective effects (16, 28-31). Whether alterations of the milk microbiome or denaturation and loss of function of milk (whey) proteins underlie these findings awaits further elucidation.

Exposure to animal sheds during early years showed an inverse, albeit non-significant effect on hay fever. This is in contrast to previous farm studies showing stronger effects (12, 32). The discrepancy might be attributable to important differences in the definition of exposure to animal sheds used in the PASTURE study, which only assessed exposure to any animal sheds without differentiating between cows, pigs, sheep and horses. The nature of animal exposure may however matter. While exposure to cow sheds showed a significant protective effect on hay fever and asthma (12), sheep sheds and keeping of hares and rabbits were risk factors for wheezing and asthma respectively in the PARSIFAL farm study (33).
The main strength of this study is its longitudinal design, which enabled us to assess the exposures at several time points before the assessment of the outcome. Excluding children with parental asthma and/or atopy and who were avoiding milk or milk products

showed similar inverse associations with hay fever consequently arguing against confounding by reverse causation. An elevated risk of diarrhea and farm milk consumption at 10.5 years was not observed (data not shown). The results of the present study show protective association of continuous consumption farm milk on hay fever. However, one of the potential caveats of the observation study is finding causality. Hence, the Milk Against Respiratory Tract Infections and Asthma (MARTHA) an ongoing interventional trial is being carried out to evaluate the preventive effect of minimally treated, i.e. only pasteurized and thus microbiologically safe cow's milk on upper respiratory tract infections and allergy (34). Further, the NNT in our study was 7, however, this study is not a randomized placebo-controlled double-blind trail and thus numbers must be taken with some caution. One of the drawbacks of the study is the missing data on hay fever at 10.5 years. However, comparing the baseline characteristics between included and excluded children did not show any significant difference except for maternal age at pregnancy, maternal smoking, parental education, and premature birth. However, adjusting for these variables did not change the results (data not shown). Another drawback is the small number in the "only early" and "only late" exposure groups that shows protective non-statistical significant effect on hay fever. However, using the RMLCA approach our study could identify these small groups manifesting that these types of habits i.e. farm milk consumption or exposure to animal sheds do exist. We performed a posthoc power calculation using SAS and considering α=0.05 (two-sided). For our sample size of 650, i.e. in the exposure groups 'continuous consumption of farm milk' and 'no consumption of farm milk' the power of study is over 80% assuming the response probabilities ranging from 0.02-0.18 for having hay fever in

children who consume farm milk and unadjusted OR of 0.24. Thus, our study was well powered to detect a relatively strong effect of farm milk consumption on hay fever.

In summary, the results of the present study demonstrate that continuous exposure of the main determinant, i.e. farm milk consumption but neither only early nor only late exposure alone conferred protection from hay fever development. The early compositional structure of the gut microbiome at age 1 year, but not age 2 month, did however in part mediate this protective effect. One might speculate that continuous consumption of unprocessed cow's milk may also increase gut microbiome richness at later ages, but we do not have data to support this notion. Overall, the findings presented herein do not support the notion of an early-determined trajectory where only early exposures in the first months of life would govern later outcomes. These results emphasize the preventive potential of continuously drinking unprocessed farm milk for hay fever protection. However, the risks associated with raw cow's milk consumption prohibit its recommendation for daily life. The results of the MARTHA trial however will shed light on potential side effects (34). Further clinical trials based on the present results are warranted.

Acknowledgments:

We thank David A. Mills, Diana Taft and Karen Kalanetra from the Department of Food Science & Technology, University of California, Davis, USA for their contribution in the generation of the original microbiome sequence and analysis.

1. World Allergy Organization (WAO): World Allergy Week 2016
Pollen Allergies – Adapting to a Changing Climate
2. Tkacz JP, Rance K, Waddell D, Aagren M, Hammerby E. Real-world evidence costs of allergic rhinitis and allergy immunotherapy in the commercially insured United States population. Curr Med Res Opin. 2021;37(6):957-65.
3. Zuberbier T, Lotvall J, Simoens S, Subramanian SV, Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2) LEN review. Allergy. 2014;69(10):1275-9.
4. von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. Nat Rev Immunol. 2010;10(12):861-8.
5. Genuneit J. Exposure to farming environments in childhood and asthma and wheeze in rural populations: a systematic review with meta-analysis. Pediatr Allergy Immunol. 2012;23(6):509-18.
6. Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, Jenmalm MC. Low diversity of the gut microbiota in infants with atopic eczema. J Allergy Clin Immunol. 2012;129(2):434-40, 40 e1-2.
7. Azad MB, Konya T, Guttman DS, Field CJ, Sears MR, HayGlass KT, et al. Infant gut microbiota and food sensitization: associations in the first year of life. Clin Exp Allergy. 2015;45(3):632-43.
8. Boutin RCT, Sbihi H, Dsouza M, Malhotra R, Petersen C, Dai D, et al. Mining the infant gut microbiota for therapeutic targets against atopic disease. Allergy. 2020;75(8):2065-8.
9. Chen CC, Chen KJ, Kong MS, Chang HJ, Huang JL. Alterations in the gut microbiotas of children with food sensitization in early life. Pediatr Allergy Immunol. 2016;27(3):254-62.
10. Depner M, Taft DH, Kirjavainen PV, Kalanetra KM, Karvonen AM, Peschel S, et al. Maturation of the gut microbiome during the first year of life contributes to the protective farm effect on childhood asthma. Nat Med. 2020;26(11):1766-75.
11. Stokholm J, Blaser MJ, Thorsen J, Rasmussen MA, Waage J, Vinding RK, et al. Maturation of the gut microbiome and risk of asthma in childhood. Nat Commun. 2018;9(1):141.
12. Illi S, Depner M, Genuneit J, Horak E, Loss G, Strunz-Lehner C, et al. Protection from childhood asthma and allergy in Alpine farm environments-the GABRIEL Advanced Studies. J Allergy Clin Immunol. 2012;129(6):1470-7 e6.
13. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, et al. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. J Allergy Clin Immunol. 2011;128(4):766-73 e4.
14. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, et al. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. Clin Exp Allergy. 2007;37(5):661-70.
15. Braun-Fahrlander C, von Mutius E. Can farm milk consumption prevent allergic diseases? Clin Exp Allergy. 2011;41(1):29-35.
16. Loss G, Depner M, Ulfman LH, van Neerven RJ, Hose AJ, Genuneit J, et al. Consumption of unprocessed cow's milk protects infants from common respiratory infections. J Allergy Clin Immunol. 2015;135(1):56-62.
17. von Mutius E, Schmid S, Group PS. The PASTURE project: EU support for the improvement of knowledge about risk factors and preventive factors for atopy in Europe. Allergy. 2006;61(4):407-13.

18. Hose AJ, Depner M, Illi S, Lau S, Keil T, Wahn U, et al. Latent class analysis reveals clinically relevant atopy phenotypes in 2 birth cohorts. J Allergy Clin Immunol. 2017;139(6):1935-45 e12.
19. Chauveau A, Dalphin ML, Mauny F, Kaulek V, Schmausser-Hechfellner E, Renz H, et al. Skin prick tests and specific IgE in 10-year-old children: Agreement and association with allergic diseases. Allergy. 2017;72(9):1365-73.
20. Roduit C, Frei R, Ferstl R, Loeliger S, Westermann P, Rhyner C, et al. High levels of butyrate and propionate in early life are associated with protection against atopy. Allergy. 2019;74(4):799-809.
21. Dostal A, Baumgartner J, Riesen N, Chassard C, Smuts CM, Zimmermann MB, et al. Effects of iron supplementation on dominant bacterial groups in the gut, faecal SCFA and gut inflammation: a randomised, placebo-controlled intervention trial in South African children. Br J Nutr. 2014;112(4):547-56.
22. A P. How to calculate the Number Needed to Treat (NNT) from Cohen's d or Hedges'g. Retrieved from https://rpubs.com/RatherBit. 2015.
23. Muthén LKM, B.O. Mplus User's Guide 7th edition (Muthén & Muthén, 1998-2012).
24. Tanaka M, Nakayama J. Development of the gut microbiota in infancy and its impact on health in later life. Allergol Int. 2017;66(4):515-22.
25. Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, Jenmalm MC. Low gut microbiota diversity in early infancy precedes asthma at school age. Clin Exp Allergy. 2014;44(6):842-50.
26. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 2012;486(7402):222-7.
27. Netea MG, Dominguez-Andres J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. Nat Rev Immunol. 2020;20(6):375-88.
28. Abbring S, Verheijden KAT, Diks MAP, Leusink-Muis A, Hols G, Baars T, et al. Raw Cow's Milk Prevents the Development of Airway Inflammation in a Murine House Dust Mite-Induced Asthma Model. Front Immunol. 2017;8:1045.
29. Abbring S, Wolf J, Ayechu-Muruzabal V, Diks MAP, Alhamwe BA, Alhamdan F, et al. Raw Cow's Milk Reduces Allergic Symptoms in a Murine Model for Food Allergy-A Potential Role For Epigenetic Modifications. Nutrients. 2019;11(8).
30. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, et al. omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. J Allergy Clin Immunol. 2016;137(6):1699-706 e13.
31. Sozanska B. Raw Cow's Milk and Its Protective Effect on Allergies and Asthma. Nutrients. 2019;11(2).
32. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet. 2001;358(9288):1129-33.
33. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, et al. Not all farming environments protect against the development of asthma and wheeze in children. J Allergy Clin Immunol. 2007;119(5):1140-7.
34. Brick T, Hettinga K, Kirchner B, Pfaffl MW, Ege MJ. The Beneficial Effect of Farm Milk Consumption on Asthma, Allergies, and Infections: From Meta-Analysis of Evidence to Clinical Trial. J Allergy Clin Immunol Pract. 2020;8(3):878-89 e3.

625

626 **Figure legends**

627 **Figure 1**. Types of exposure classes.

628 Solution for repeated measure latent classes defined by different exposures, which are a)
629 exposure to animal sheds, and b) farm milk consumption in the PASTURE children. Numbers in
630 parentheses indicate the total number of children in each class.

631 **Figure 2.** Associations of farm milk exposure classes with hay fever at age 10.5 years.

632 Associations of farm milk exposure classes with hay fever at age 10.5 years. Models are adjusted
633 for centers, growing up on a farm, and parental atopy. The forest plot represent the adjusted odds
634 ratios (aOR) with 95%confidence intervals [95%CI].

635 **Figure 3**. Association of gut microbiome richness, and Shannon-index at the age of 2 and 12
636 months with hay fever at 10.5 years.

637 Association of gut microbiome richness, and Shannon-index at months 2 (hay fever/total:
638 59/439) and 12 (hay fever/total: 79/633) with hay fever at 10.5 years. Models are adjusted for
639 centers, growing up on a farm, and parental atopy. The association with hay fever is shown as
640 aOR per-interquartile-range of the probability along with 95%CI.

641 **Figure 4.** Association of consumption of farm milk, consumption of processed milk, and
642 exposure to animal sheds in infancy with gut microbiome richness at month 12.

643 Association of consumption of farm milk (N=624), consumption of processed milk (N=624) and
644 exposure to animal sheds (N=617) with richness at 12 months. Models are adjusted for centers,

growing up on a farm, and parental atopy. The forest plot represent the adjusted geometric mean ratios with 95%CI.

Figure 5. Mediation analysis.

Mediation analysis of the protective effect of consumption of farm milk and exposure to animal sheds in infancy on hay fever mediated by gut microbiome richness at 12 months adjusting for centers (N=466). The figure shows the direct (β_1), indirect (β_2) and total (β) effects as well as their respective 95% CI from the path model. The proportion of the mediated (indirect) effect was 18.4%.

653 **Table 1:** Description of the study population

Characteristic	All (N=769)	Hay fever (N=99 (12.9%))	No hay fever (N=670 (87.1%))	*P value*
	N (%)/Total	N (%)/Total	N (%)/Total	
Farm child (yes)	367 (47.7)/768	31 (31.3)/99	336 (50.2)/670	*0.0005*
Exposure to cats at age of 2 months (yes)	199 (26.0)/767	19 (19.2)/99	180 (27.0)/668	*0.11*
Exposure to dogs at age of 2 months (yes)	147 (19.2)/766	17 (17.2)/99	130 (19.5)/667	*0.68*
Maternal age at pregnancy (years) †	31.2±4.5 (N=769)	31.4±4.4 (N=99)	31.2±4.5 (N=670)	*0.52*
Maternal smoking (yes)	96 (12.5)/766	16 (16.5)/97	80 (12.0)/669	*0.25*
Second hand smoking (yes)	33 (4.3)/764	3 (3.1)/98	30 (4.5)/666	*0.79*
Parental education (yes)				*0.13*
Low	62 (8.1)/764	3 (3.1)/97	59 (8.9)/667	
Medium	280 (36.7/764)	39 (40.2)/97	241 (36.1)/667	
High	422 (56.7)/764	55 (56.7)/97	367 (55.0)/667	
Use of antibiotics during pregnancy (yes)	204 (27.0)/755	26 (26.5)/98	178 (27.1)/657	*1.00*

Parental atopy (yes)	416 (54.4)/765	72 (73.5)/98	344 (51.6)/667	*<0.0001*
Mode of delivery (normal)	624 (81.9)/762	82 (83.7)/98	542 (81.6)/664	*0.68*
Premature birth (yes)	11 (1.4)/769	1 (1.0)/99	10 (1.5)/670	*1.00*
Birth weight (kg) †	3.4±0.44 (N=605)	3.4±0.5 (N=82)	3.4±0.4 (N=523)	*0.81*
Breast feeding 2 months (yes)	711 (92.7)/767	90 (90.9)/99	621 (93.0)/668	*0.41*
Gender (female)	366 (47.7)/768	42 (42.4)/99	324 (48.4)/669	*0.28*
Having siblings (yes)	494 (64.2)/769	60 (60.6)/99	434 (64.8)/670	*0.43*
Use of antibiotics during first year of life (weeks) †	0.03±0.3 (N=746)	0.01±0.1 (N=97)	0.03±0.4 (N=649)	*0.86*
Doctor‘s diagnosis of hay fever (yes)	36 (4.7)/769	36 (36.4)/99	NA	*NA*
Inhalant sensitization (IgE≥0.7 kU/L or SPT≥3mm) at 10.5 years	259 (49.6)/522	66 (86.8)/76*	193 (43.3)/446*	*<0.0001*
Concomitants				
Asthma (yes)	69 (9.0)/764	28 (28.9)/97	41 (6.2)/667	*<0.0001*
Eczema (yes)	100 (13.1)/763	36 (36.7)/98	64 (9.6)/665	*<0.0001*
Food allergy (yes)	41 (5.5)/746	21 (21.7)/97	20 (3.1) /649	*<0.0001*

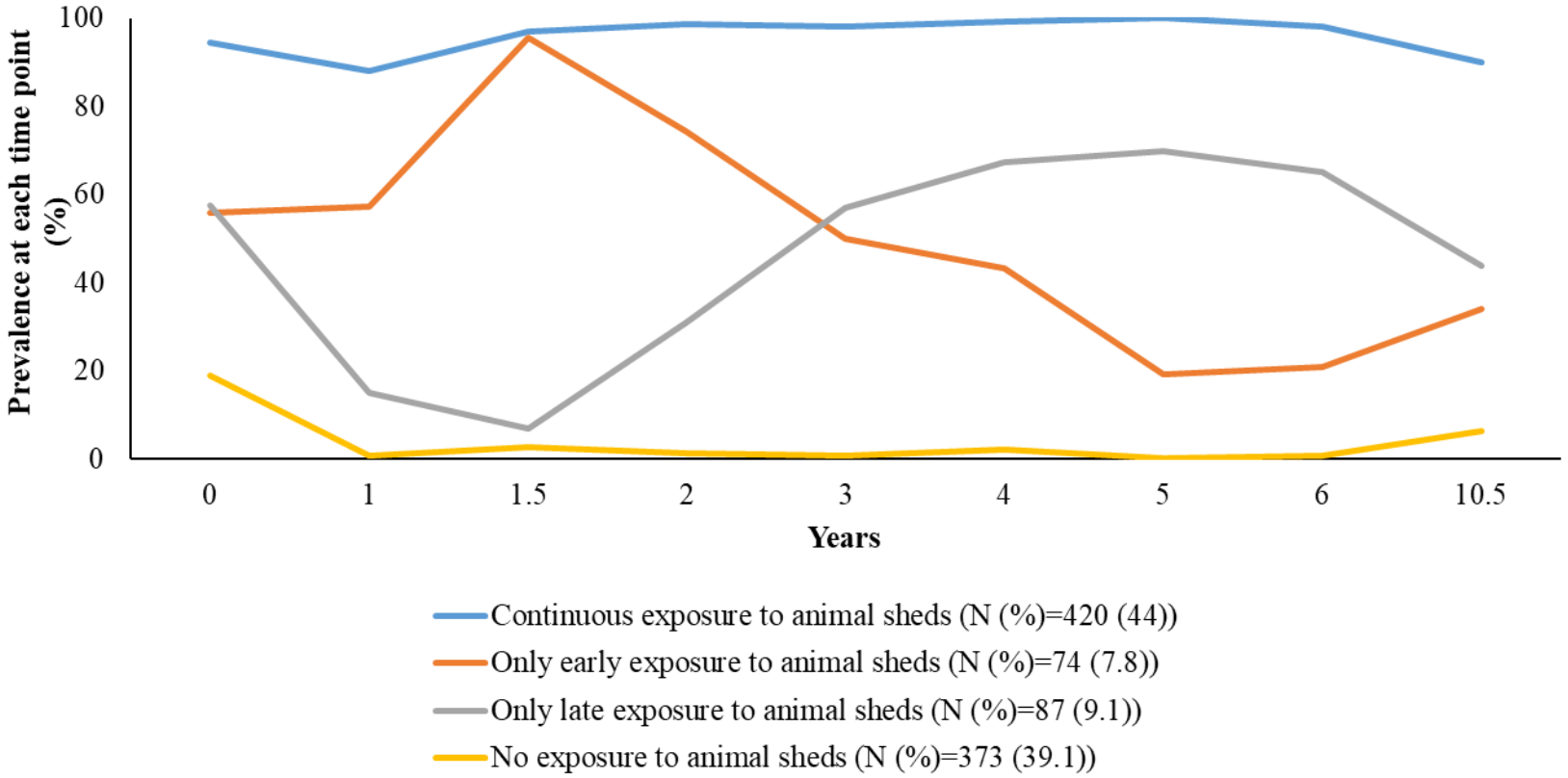
The categorical variables are presented as frequency (percentage) and the continuous variables as mean †: mean±standard deviation. The test for differences between the groups are $\chi 2$ or Fischer's Exact test for categorical variables and Mann Whitney U test for continuous variables.

Farm child was defined as “Children of mothers living on family-run livestock farms were assigned to the farm group. The non-farm group included children of mothers from the same rural areas but not living on a farm”. Exposure to pets at the age of 2 months (cats and dogs) was defined by asking “if you have cats?”, “if you have dogs?” and “if they stay indoors in the house?” . Maternal smoking during pregnancy was defined using the following questions “Have you in your life smoked more than 5 packs of cigarettes?” Or “Have you quit smoking in the meantime?” and if yes “Was it during this pregnancy?”. Smoking by father, “Have you in your life smoked more than 5 packs of cigarettes?” Or “Do you still smoke?”. Second hand smoking “How many cigarettes are on average per day were smoked in your house by other people?” If greater than 1 then second hand smoking was defined as 1 else 0. Parental education was defined as low (less than 10 years), medium (10 years) and high (greater than 10 years). Parental atopy was defined as doctor’s diagnosis of hay fever, atopic dermatitis, or asthma ever in mother or father. Use of antibiotics during pregnancy was defined by asking “Have you taken antibiotics since the beginning of pregnancy?” Or “Have you taken any antibiotics during this pregnancy?”. Child was defined as premature if the child was born before the completion of 37 weeks of pregnancy. Use of antibiotics by a child during first year of life was defined as “Total No. of weeks with antibiotics ingested”. Breastfeeding at the age of 2 months (yes or no) was defined by asking “if you have ever breastfed?”. SPT: skin prick test. Inhalant sensitization was defined as at least one IgE specific to alder, birch, hazel, plantain, mugwort, alternaria, grass, rye, *Dermatophagoides pteronyssinus*, *Dermatophagoides farina, cat, dog, or horse* at levels $\geq 0.7 IUml^{-1}$ or SPT (birch, grass, alternaria, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, or dog) ≥3mm. Serum specific IgE and SPT was not measured in the Austrian study center, hence only sub-sample N=522 was included.. Asthma was defined as a physician’s diagnosis of asthma or recurrent obstructive bronchitis established until 10.5 years. Eczema and food allergy were defined as physician diagnoses at least once until the age of 10.5 years. NA: not applicable.

Figure No. - Unmarked1

Figure 1.

a)



b)

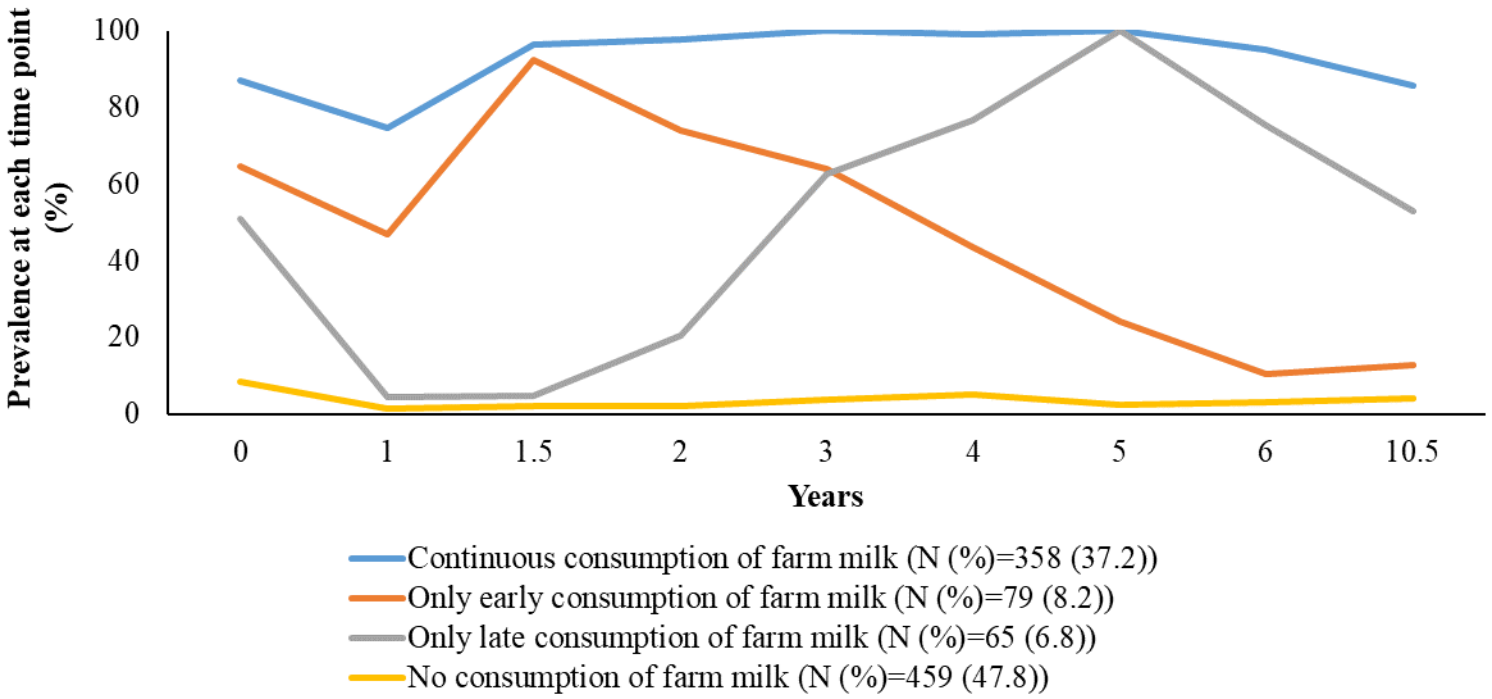

Prevalence at each time point (%)
100
80
60
40
20
0
0
1
1.5
2
3
4
5
6
10.5
Years
Continuous consumption of farm milk (N (%)=358 (37.2))
Only early consumption of farm milk (N (%)=79 (8.2))
Only late consumption of farm milk (N (%)=65 (6.8))
No consumption of farm milk (N (%)=459 (47.8))

Figure No. - Unmarked2

Figure 2

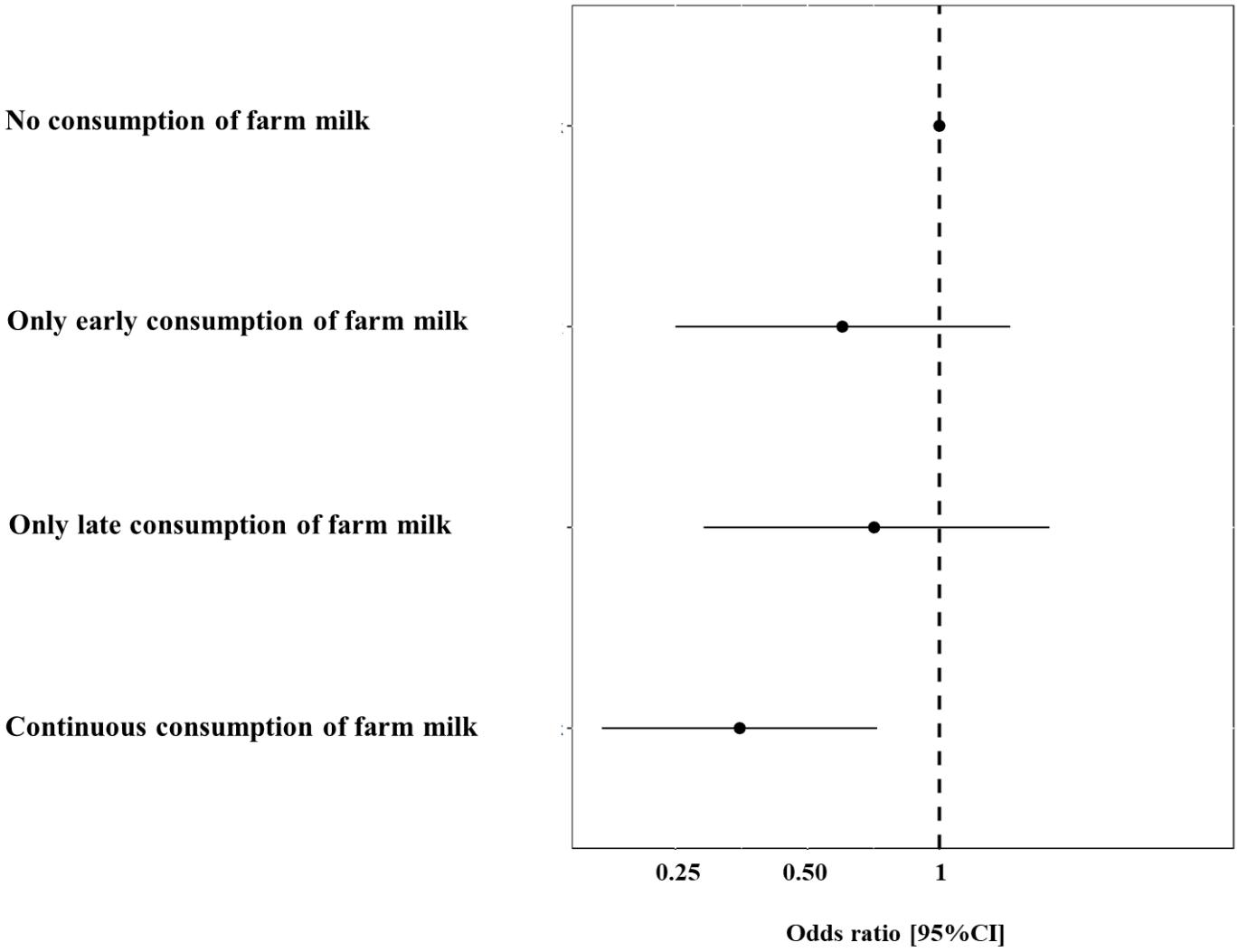


Figure No. - Unmarked3

Figure 3

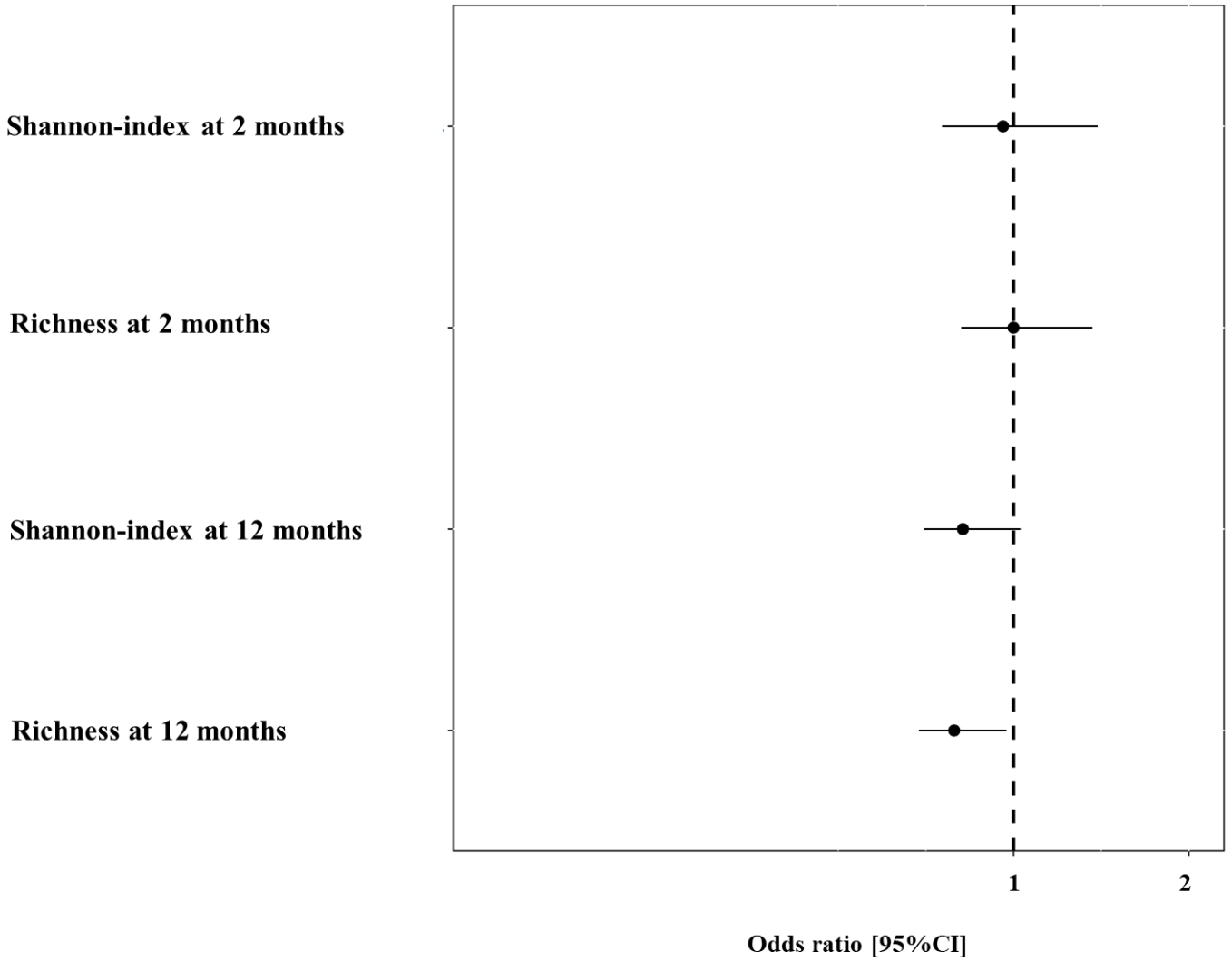


Figure No. - Unmarked4

Figure 4.

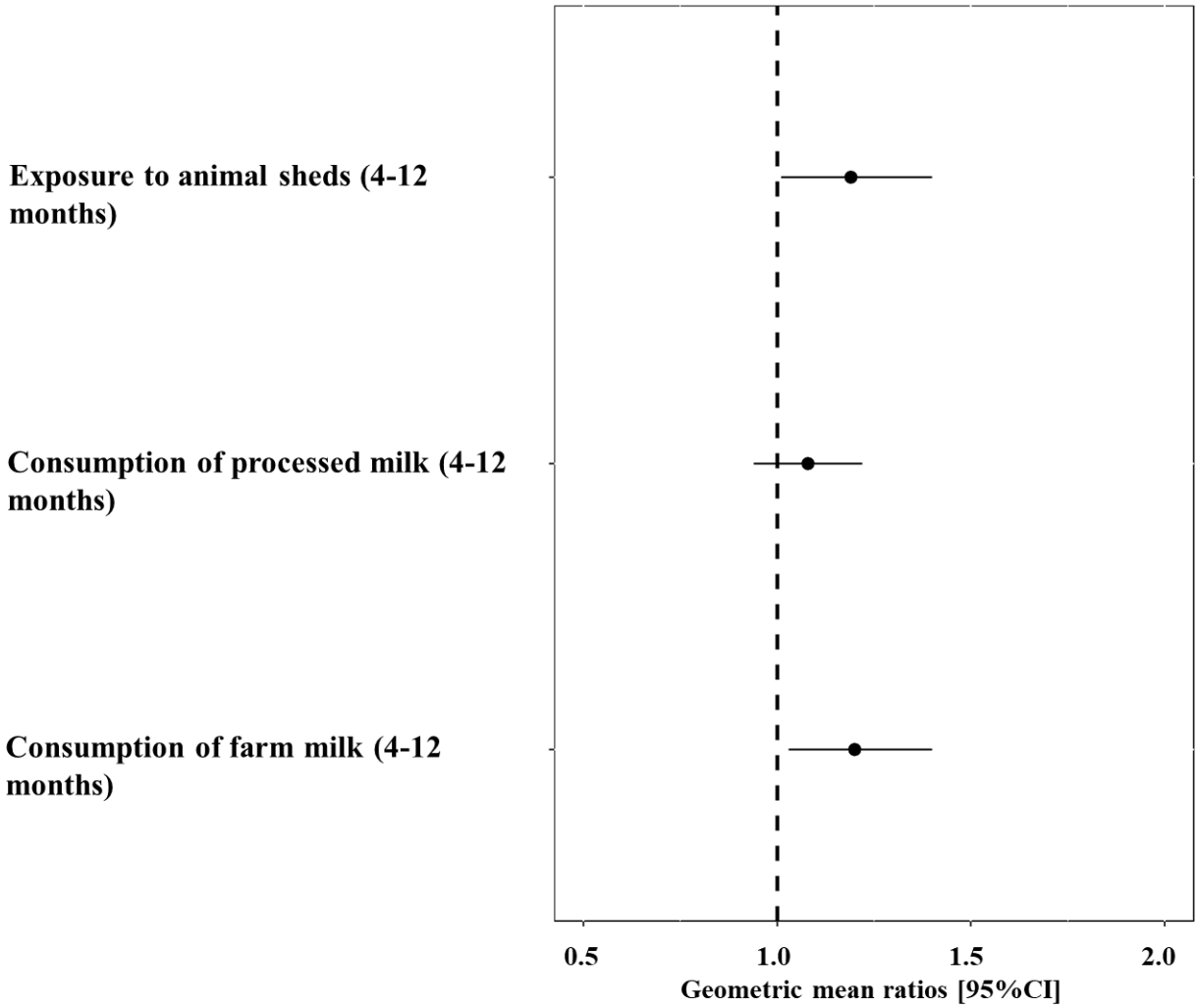
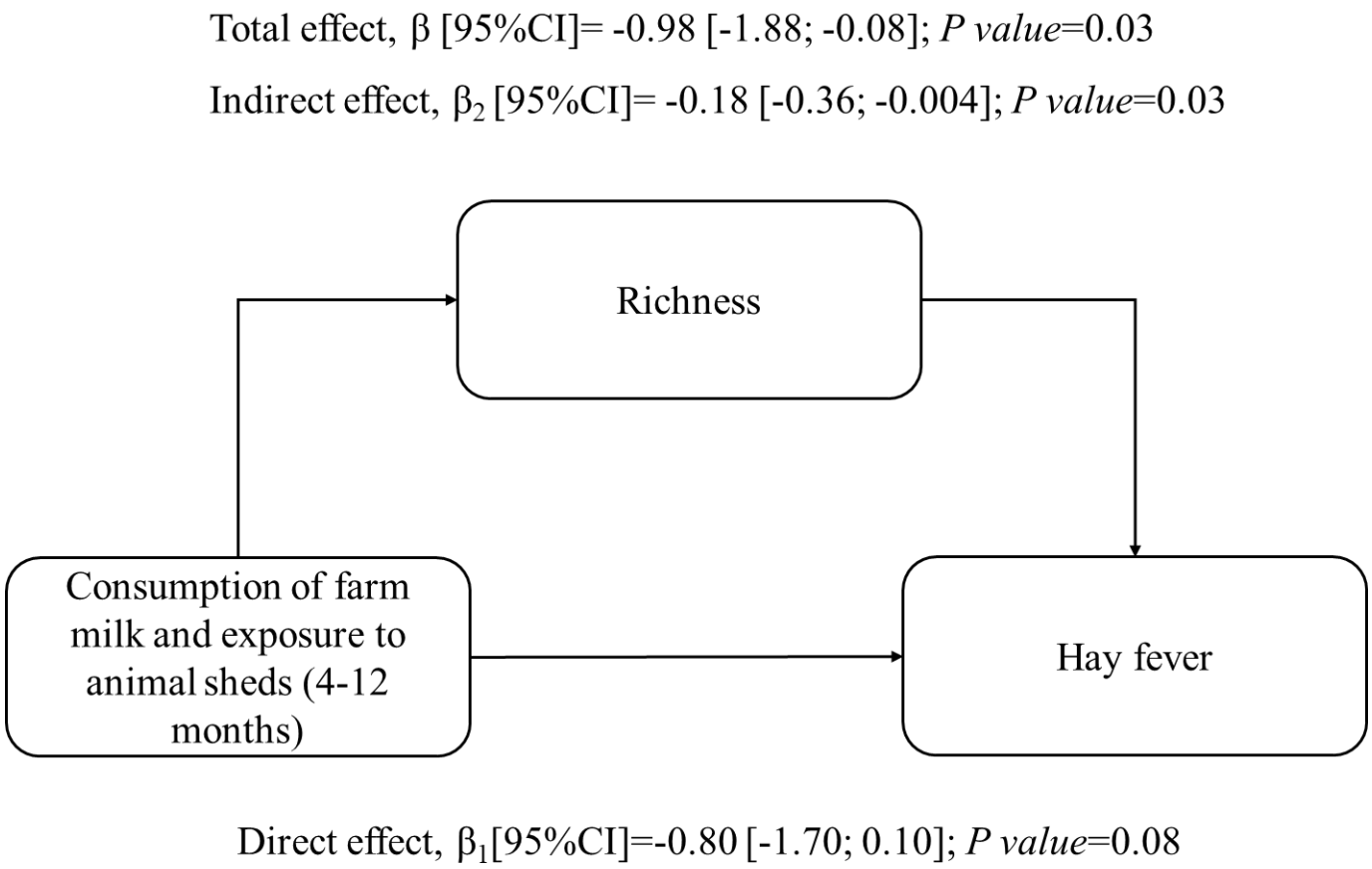


Figure 5.


Total effect, β [95%CI]= -0.98 [-1.88; -0.08]; *P value*=0.03
Indirect effect, β_2 [95%CI]= -0.18 [-0.36; -0.004]; *P value*=0.03
Richness
Consumption of farm milk and exposure to animal sheds (4-12 months)
Hay fever
Direct effect, β_1[95%CI]=-0.80 [-1.70; 0.10]; *P value*=0.08

Online Repository Text

Continuous rather than solely early farm exposure protect from hay fever development.

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Methods:

Questionnaires:

Information were collected through mothers using questionnaires in interviews or self-administered questionnaires within the third trimester of pregnancy and when the children were 2, 12, 18 months of age and then at the age of 2, 3, 4, 5, 6, and 10.5 years. Using weekly and monthly diaries and questionnaires from the 8th to 53rd weeks of age, additional information on child's health, nutrition and farm-related exposures were collected (E1, E2).

Definitions of outcome:

Inhalant sensitization at 10.5 years was defined as at least one IgE specific to alder, birch, hazel, plantain, mugwort, alternaria, grass, rye, *Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, cat, dog, or horse at levels $\geq 0.7 IUml^{-1}$ or SPT (birch, grass, alternaria, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, or dog) ≥3mm. Serum specific IgE and SPT was not measured in the Austrian study center. Serum specific IgE was assessed using the semiquantitative Allergy Screen test panel for atopy (Mediwiss Analytic, Moers; Germany) (E3). As described before, SPTs were performed on the anterior part of the forearm using a Stallerpoint ® (Stallergenes, Antony, France) (E4). Incident hay fever at 10.5 years (N=48) was defined by parent reported symptoms (itchy, runny, or blocked nose without a cold accompanied by red itchy eyes) and/or a physician's diagnosis of hay fever in the last 12 months using questionnaires at age 10.5 years and excluding those having hay fever before the age of 10.5 years.

Assessment of exposures:

Socioeconomic and lifestyle factors, farm-related exposures, health status of women, their husbands and their children were assessed through questionnaires in interviews or self-administered questionnaires to the mothers within the third trimester of pregnancy and when the children were 2, 12, 18 months of age and then at the age of 2, 3, 4, 5, 6, and 10.5 years. Maternal smoking during pregnancy was defined using the following questions “Have you in your life smoked more than 5 packs of cigarettes?” Or “Have you quit smoking in the meantime?” and if yes “Was it during this pregnancy?”. Smoking by father, “Have you in your life smoked more than 5 packs of cigarettes?” Or “Do you still smoke?”. Second hand smoking was defined by asking “How many cigarettes are on average per day were smoked in your house by other people?” If greater than one then second hand smoking was defined as 1 else 0. Parental education was defined as low (less than 10 years), medium (10 years) and high (greater than 10 years). Parental atopy (yes or no) was defined as doctor’s diagnosis of hay fever, atopic dermatitis, or asthma ever in mother or father. Use of antibiotics during pregnancy was defined by asking “Have you taken antibiotics since the beginning of pregnancy?” Or “Have you taken any antibiotics during this pregnancy?”. Child was defined as premature if the child was born before the completion of 37 weeks of pregnancy. Use of antibiotics by a child during first year of life was defined as “Total number of weeks with antibiotics ingested”. Further, breastfeeding at age of 2 months (yes or no) was defined by asking “if you have ever breastfed?”, exposure to pets at age of 2 months (cats and dogs) was defined by asking “if you have cats?”, “if you have dogs?” and “if they stay indoors in the house?”, and data on having siblings (yes or no) were also collected. Further, asthma was defined as a physician’s diagnosis of asthma or recurrent

obstructive bronchitis established until 10.5 years. Eczema and food allergy were defined as physician diagnoses at least once until the age of 10.5 years.

Additionally, the frequency of farm milk consumption at each time point by a child (daily, 1-6 times a week, less than once a week or no consumption) was further weighted as follows: weight of 3 was assigned for daily consumption, a weight of 2 for 1-6 times a week, a weight of 1 for consumption less than once a week and 0 for no consumption). The weights over the years were then summed up as farm milk consumption score representing the frequency of farm milk consumed. Since data on frequency of processed milk consumption was available only at age 10.5 years, instead of constructing a score it was categorized as daily, 1-6 times a week and no (less than once a week or no) consumption of processed milk.

DNA extraction from fecal samples and sequencing analyses:

Briefly, the fecal samples were frozen within 10 minutes of collection, and stored at -20°C until further processing. Targeted DNA amplifications using primers targeting the V4 region of the 16S rRNA gene were performed. The amplicon sequencing was done on Illumina MiSeq instrument producing 250-bp paired end sequences as described previously (E5). Sequencing processing was done using QIIME2-2018.6 (Quantitative Insights Into Microbial Ecology) and reads were denoised using DADA2 (E6, E7). Samples were rarefied at the minimum sequence numbers 1,029. Rarefaction and calculation of richness and Shannon-index was iterated 1,000 times and the resulting measures of α-diversity were then averaged (E5). As described previously, SCFA levels were modeled by the relative abundance of bacterial genera in children with available SCFA measurements using the “predict” function of R-package ranger (E5). These prediction models were then applied to predict SCFA production scores (butyrate and propionate) in the entire population.

Statistical analyses:

Repeated measure latent classes (exposure classes)

Using farm milk consumption and processed milk consumption exposures together, 3 types of farm and processed milk exposure classes were identified: i) 'high farm and low processed milk', ii) 'mixed consumption of farm and processed milk', and iii) 'low farm and high processed milk' (Figure E2(a)). The children were allocated to specific exposure classes by their highest posterior probabilities. The optimal number of exposure classes was then determined according to the Bayesian Information Criterion. Further, the labelling of the exposure classes was based on main features of each class. The analyses were done on children having data at least at 7 of the 8 assessed time points for the combined farm and processed milk consumption.

The associations between hay fever and farm and shop milk consumption exposure classes was assessed by logistic regression. The above model was adjusted for centers and confounders, (growing up on a farm, and parental asthma and/or atopy). We tested the differences in relative abundance of most common single bacterial genera at 2 and 12 months with hay fever by Wilcoxon test, main associations ($p<0.05$) were then confirmed in logistic regression models using center-log-ratio-transformed variables. Gut microbiome richness and Shannon-index at 2 and 12 months were transformed by dividing the original variable by their respective interquartile range (IQR: IQRrichness_2m: 8.07, IQRShannon-index_2m: 0.75, IQRrichness_12m: 15.9 and IQRShannon-index_12m: 0.75) and the new variables were then included in the regression models (logistic regression to test the association with outcome hay fever at 10.5 years and linear regression to test the associations between consumption of farm

145 milk, consumption of processed milk and exposure to animal sheds in infancy). The association
146 with hay fever is then represented as adjusted odds ratio per IQR of the probability.

E1. Loss G, Depner M, Ulfman LH, van Neerven RJ, Hose AJ, Genuneit J, et al. Consumption of unprocessed cow's milk protects infants from common respiratory infections. J Allergy Clin Immunol. 2015;135(1):56-62.

E2. Loss GJ, Depner M, Hose AJ, Genuneit J, Karvonen AM, Hyvarinen A, et al. The Early Development of Wheeze. Environmental Determinants and Genetic Susceptibility at 17q21. Am J Respir Crit Care Med. 2016;193(8):889-97.

E3. Depner M, Ege MJ, Genuneit J, Pekkanen J, Roponen M, Hirvonen MR, et al. Atopic sensitization in the first year of life. J Allergy Clin Immunol. 2013;131(3):781-8.

E4. Chauveau A, Dalphin ML, Kaulek V, Roduit C, Pugin A, von Mutius E, et al. Disagreement between Skin Prick Tests and Specific IgE in Early Childhood. Int Arch Allergy Immunol. 2016;170(2):69-74.

E5. Depner M, Taft DH, Kirjavainen PV, Kalanetra KM, Karvonen AM, Peschel S, et al. Maturation of the gut microbiome during the first year of life contributes to the protective farm effect on childhood asthma. Nat Med. 2020;26(11):1766-75.

E6. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335-6.

E7. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3.

Figure E1. Proportion of farm milk consumption and exposure to animal sheds over time in the PASTURE children with data on hay fever at 10.5 years (N=769).

Figure E2. Association of farm milk consumption exposure classes with the stringent definition of hay fever

Models are adjusted for centers, growing up on a farm, and parental atopy. The forest plot represent the aOR with 95%confidence intervals [95%CI].

Figure E3. Farm and processed milk consumption exposure classes

a) Solution for repeated measure latent classes defined by farm and processed milk consumption in the PASTURE children. Numbers in parentheses indicate the total number of children in each class. b) Association of farm and processed milk consumption exposure classes with hay fever. Models are adjusted for centers, growing up on a farm, and parental atopy. The forest plot represent the aOR with 95%CI.

Figure E4. Association of the frequency of processed milk consumption at the age of 10.5 years with hay fever at 10.5 years in the PASTURE children.

Model is adjusted for centers, growing up on a farm, and parental atopy. The forest plot represent the aOR with 95%CI.

182 **Table E1.** Description of the included and excluded study population

Characteristic	Included in the study (N=769)	Excluded in the study (N=364)	*P value*
	N (%)/Total	N (%)/Total	
Farm child (yes)	367 (47.7)/769	164 (45.0)/364	*0.41*
Exposure to cats at age of 2 months (yes)	199 (26.0)/767	82 (25.9)/323	*0.88*
Exposure to dogs at age of 2 months (yes)	147 (19.2)/766	48(14.9)/322	*0.10*
Maternal age at pregnancy (years) †	31.3±4.5 (N=769)	30.2±5.0 (N=364)	*<0.003*
Maternal smoking (yes)	96 (12.5)/766	62 (17.0)/363	*0.04*
Second hand smoking (yes)	33 (4.3)/764	16 (5.0)/322	*0.63*
Parental education (yes)			
Low	62 (8.1)/764	63 (18.1)/349	
Medium	280 (36.7)/764	146 (41.8)/349	
High	422 (55.2)/764	140 (40.1)/349	*<0.001*
Use of antibiotics during pregnancy (yes)	204 (27.0)/755	85 (26.1)/326	*0.77*
Parental atopy (yes)	416 (54.4)/765	176 (52.7)/334	*0.60*

Mode of delivery (normal)	624 (81.9)/762	267 (83.4)/320	*0.66*
Premature birth (yes)	11 (1.4)/769	50 (13.7)/364	*<0.0001*
Birth weight (kg) †	3.4±0.4 (N=605)	3.4±0.44 (N=239)	*0.31*
Breast feeding 2 months (yes)	711 (92.7)/767	294 (91.0)/323	*0.27*
Gender (female)	366 (47.7)/768	166 (50.9)/326	*0.36*
Having siblings (yes)	494 (64.2)/769	227 (62.3)/364	*0.51*
Use of antibiotics during first year of life (weeks) †	0.03±0.3 (N=746)	0.02±0.1 (N=286)	*0.50*

The categorical variables are presented as frequency (percentage) and the continuous variables as †: mean±standard deviation. The test for differences between the groups are $\chi 2$ test for categorical variables and Mann Whitney U test for continuous variables. Farm child was defined as "Children of mothers living on family-run livestock farms were assigned to the farm group. The non-farm group included children of mothers from the same rural areas but not living on a farm". Exposure to pets at the age of 2 months (cats and dogs) was defined by asking "if you have cats?", "if you have dogs?" and "if they stay indoors in the house?". Maternal smoking during pregnancy was defined using the following questions "Have you in your life smoked more than 5 packs of cigarettes?" Or "Have you quit smoking in the meantime?" and if yes "Was it during this pregnancy?". Smoking by father, "Have you in your life smoked more than 5 packs of cigarettes?" Or "Do you still smoke?". Second hand smoking "How many cigarettes are on average per day were smoked in your house by other people?" If greater than 1 then second hand smoking was defined as 1 else 0. Parental education was defined as low (less than 10 years), medium (10 years) and high (greater than 10 years). Parental atopy was defined as doctor's diagnosis of hay fever, atopic dermatitis, or asthma ever in mother or father. Use of antibiotics during pregnancy was defined by asking "Have you taken antibiotics since the beginning of pregnancy?" Or "Have you taken any antibiotics during this pregnancy?". Child was defined as premature if the child was born before the completion of 37 weeks of pregnancy. Use of antibiotics by a child during first year of life was defined as "Total No. of weeks with antibiotics ingested". Breastfeeding at the age of 2 months (yes or no) was defined by asking "if you have ever breastfed?". Asthma was defined as a physician's diagnosis of asthma or recurrent obstructive bronchitis established until 10.5 years. Eczema and food allergy were defined as physician diagnoses at least once until the age of 10.5 years. NA: not applicable.

197 **Table E2**. Association between animal sheds exposure classes with hay fever at 10.5 years.

	Hay fever/Total	OR [95% CI], *P value*
Model 1		
No exposure to animal sheds	56 (18.0)/312	1
Only early exposure to animal sheds	2 (3.9)/52	0.26 [0.06; 1.15], *0.08*
Only late exposure to animal sheds	9 (12.5)/72	0.88 [0.40; 1.96], *0.76*
Continuous exposure to animal sheds	31 (9.4)/329	1.14 [0.50; 2.64], *0.75*
Model 2		
No exposure to animal sheds	56 (18.0)/312	1
Only early exposure to animal sheds	2 (3.9)/52	0.32 [0.07; 1.44], *0.14*
Only late exposure to animal sheds	9 (12.5)/72	1.04 [0.46; 2.36], *0.92*
Continuous exposure to animal sheds	31 (9.4)/329	1.94 [0.79; 4.74], *0.15*

198 Model 1: adjusted for centers, growing up on a farm, and parental atopy. Model 2: adjusted for centers, farm milk consumption exposure classes, and parental
199 atopy. The number of children included in the analyses are different to that shown in Figure 1(a) due to the missing values of hay fever at year 10.5 years.

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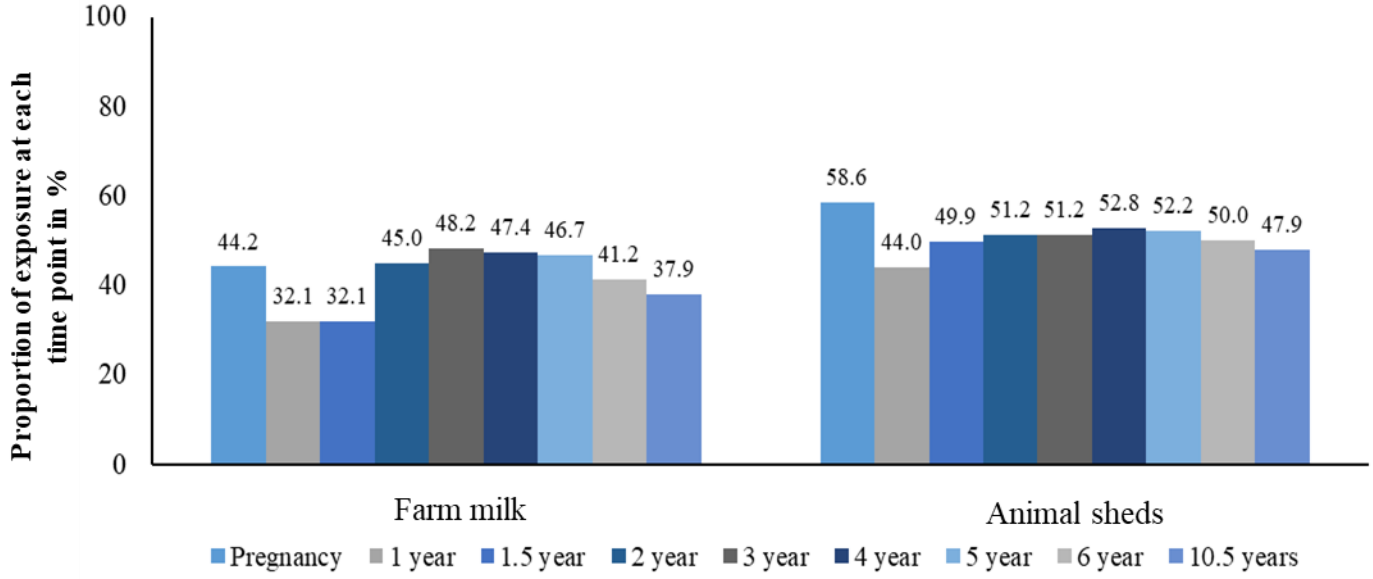
203 **Table E3**. Proportion of children in each exposure classes.

Exposure classes	Hay fever (%)/Total
Farm milk	
No consumption of farm milk	70 (18.9)/371
Only early consumption of farm milk	7 (10.9)/64
Only late consumption of farm milk	6 (11.8)/51
Continuous consumption of farm milk	15 (5.4)/279
Farm milk and processed milk	
Low farm milk and high processed milk	49 (16.2)/302
Mixed consumption of farm milk and processed milk	9 (7.1)/127
High farm milk and low processed milk	7 (4.3)/162

204 Numbers in parentheses indicate percent of children with hay fever in each exposure class. The number of children included in the analyses are different to that
205 shown in Figure 1(b) and Figure E3(a) due to the missing values of hay fever at year 10.5 years.

Figure E1.



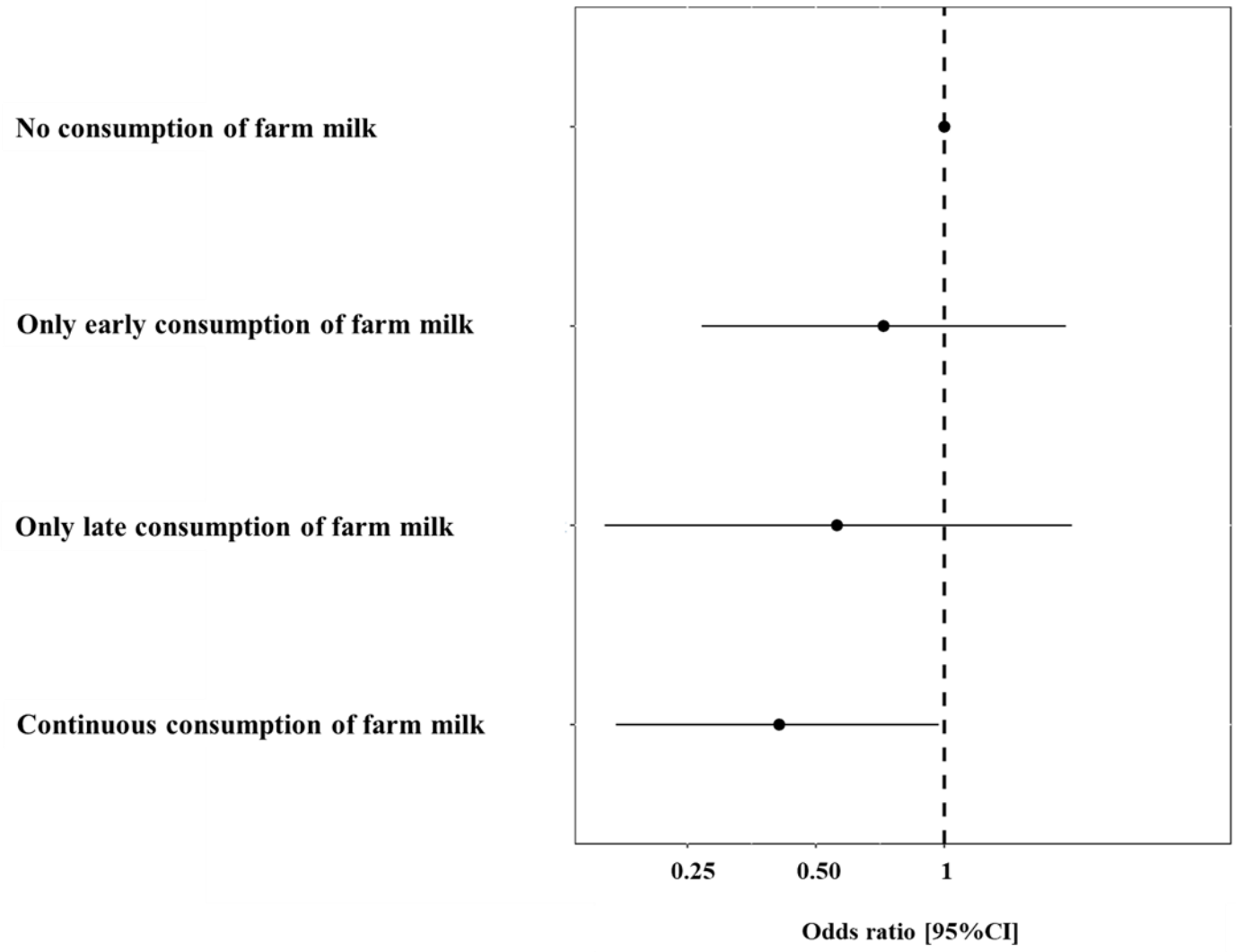
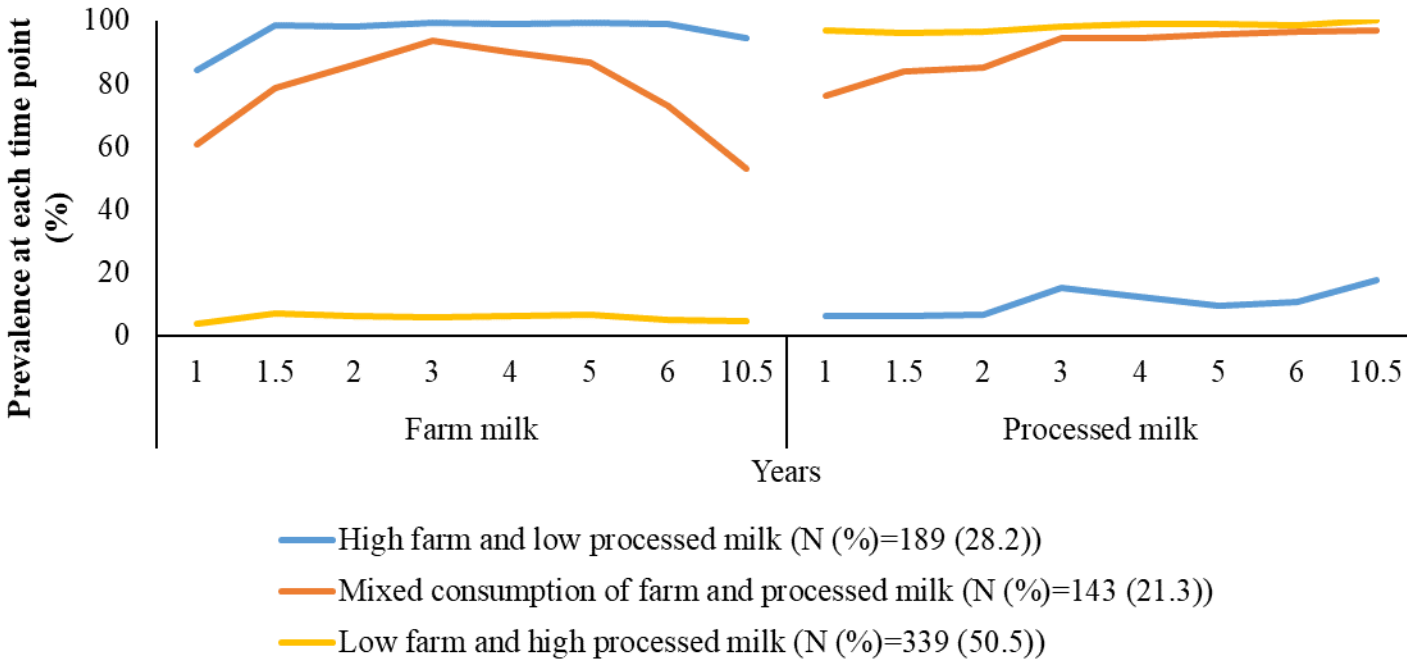


Figure E3.

(a)



(b)

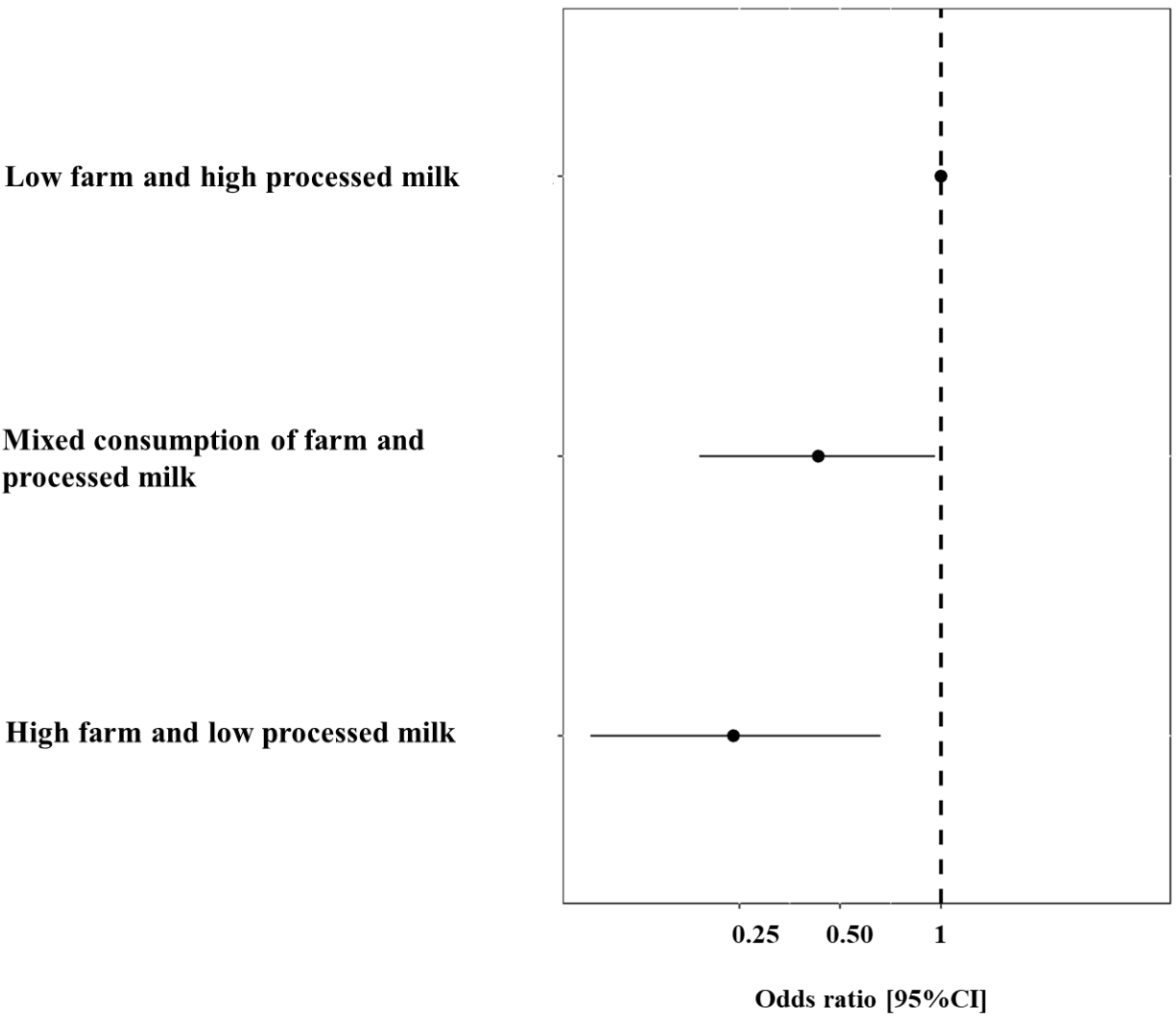
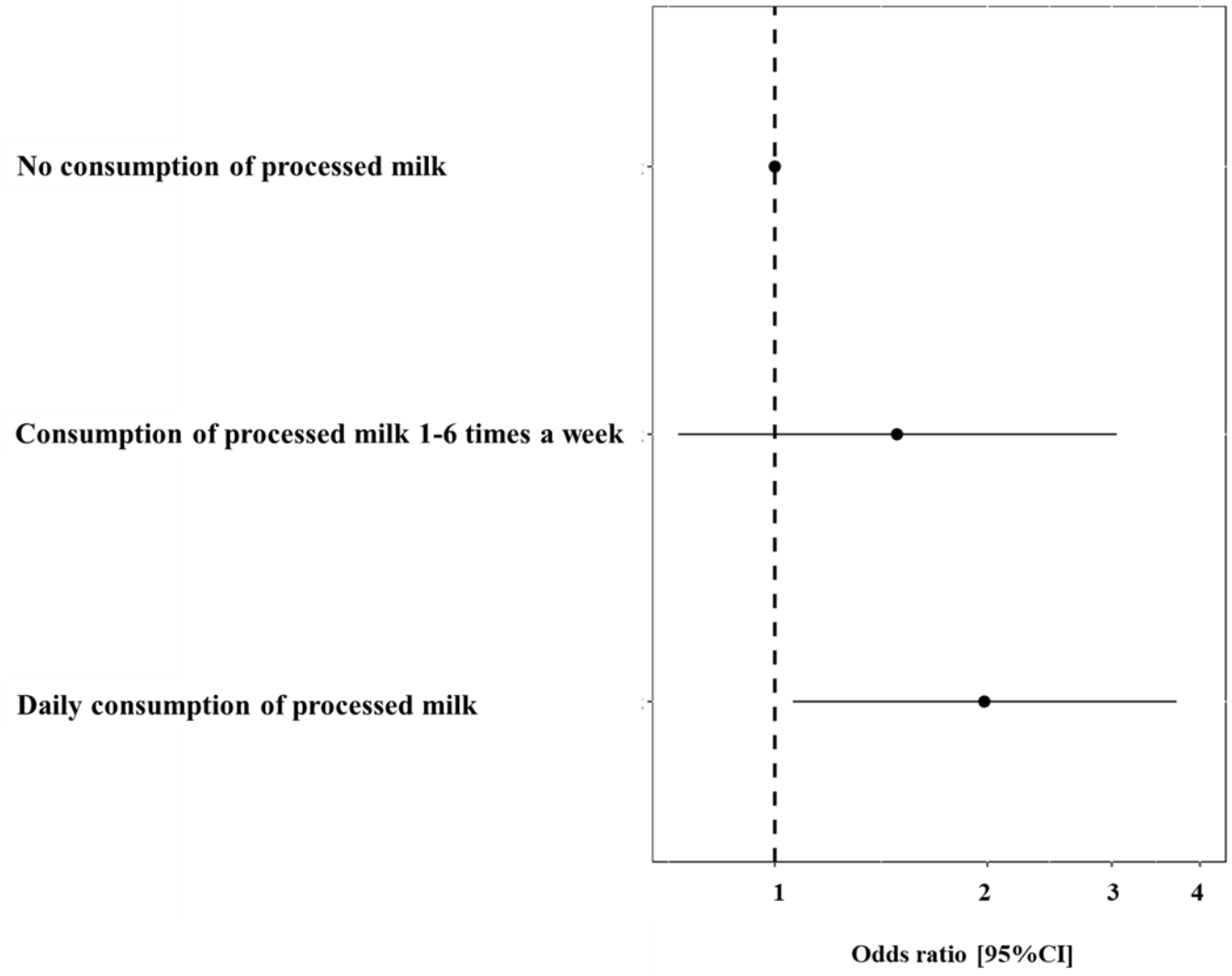
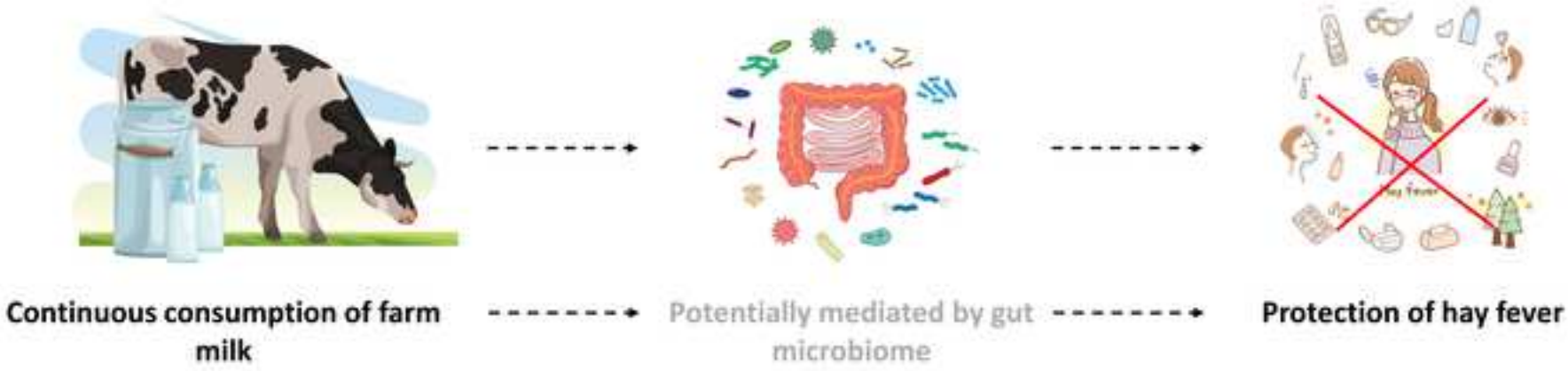

Low farm and high processed milk
Mixed consumption of farm and processed milk
High farm and low processed milk
0.25
0.50
1
Odds ratio [95%CI]

Figure E4.





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Manuscript Number (if known): INPRACTICE-D-22-00656

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		METHOD FOR TESTING A SUBJECT THOUGHT TO HAVE OR TO BE PREDISPOSED TO ASTHMA European patent application 5 EP07301135.5	
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Manuscript Title: **Continuous rather than solely early farm exposure protect from hay fever development.**

Manuscript Number (if known): INPRACTICE-D-22-00656

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Your Name: Jon Genuneit

Manuscript Title: **Continuous rather than solely early farm exposure protect from hay fever development.**

Manuscript Number (if known): INPRACTICE-D-22-00656

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		The journal Pediatric Allergy and Immunology (co-owned by Wiley and EAACI)	Personal honorarium for serving as Associate Editor
6	Payment for expert testimony	☒ **None**	
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13	Other financial or non-financial interests	☒ **None**	

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☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 10/4/2022

Your Name: Andreas Böck

Manuscript Title: **Continuous rather than solely early farm exposure protect from hay fever development.**

Manuscript Number (if known): Click or tap here to enter text.

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	☒ None	
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☒ **None**	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

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ICMJE DISCLOSURE FORM

Date: 9/27/2022

Your Name: Laurent Lucie

Manuscript Title: **Continuous rather than solely early farm exposure protect from hay fever development.**

Manuscript Number (if known): Click or tap here to enter text.

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

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ICMJE DISCLOSURE FORM

Date: 9/30/2022

Your Name: Petra Ina Pfefferle

Manuscript Title: **Continuous rather than solely early farm exposure protect from hay fever development.**

Manuscript Number (if known): Click or tap here to enter text.

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ICMJE DISCLOSURE FORM

Date: Click or tap to enter a date. 6.10.2022

Your Name: Click or tap here to enter text. Harald Renz, MD

Manuscript Title: **Continuous rather than solely early farm exposure protect from hay fever development.**

Manuscript Number (if known): Click or tap here to enter text.

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