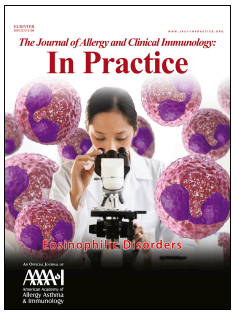

Continuous rather than solely early farm exposure protect from hay fever development.

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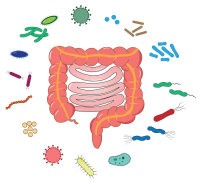
Continuous consumption of farm milk



Potentially mediated by gut microbiome

Protection of hay fever

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

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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

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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.

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

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 frequency of farm milk consumption was assessed at the age of 18 months, 2, 3, 4, 5, 6 and 10.5 years of age. 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 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.

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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*

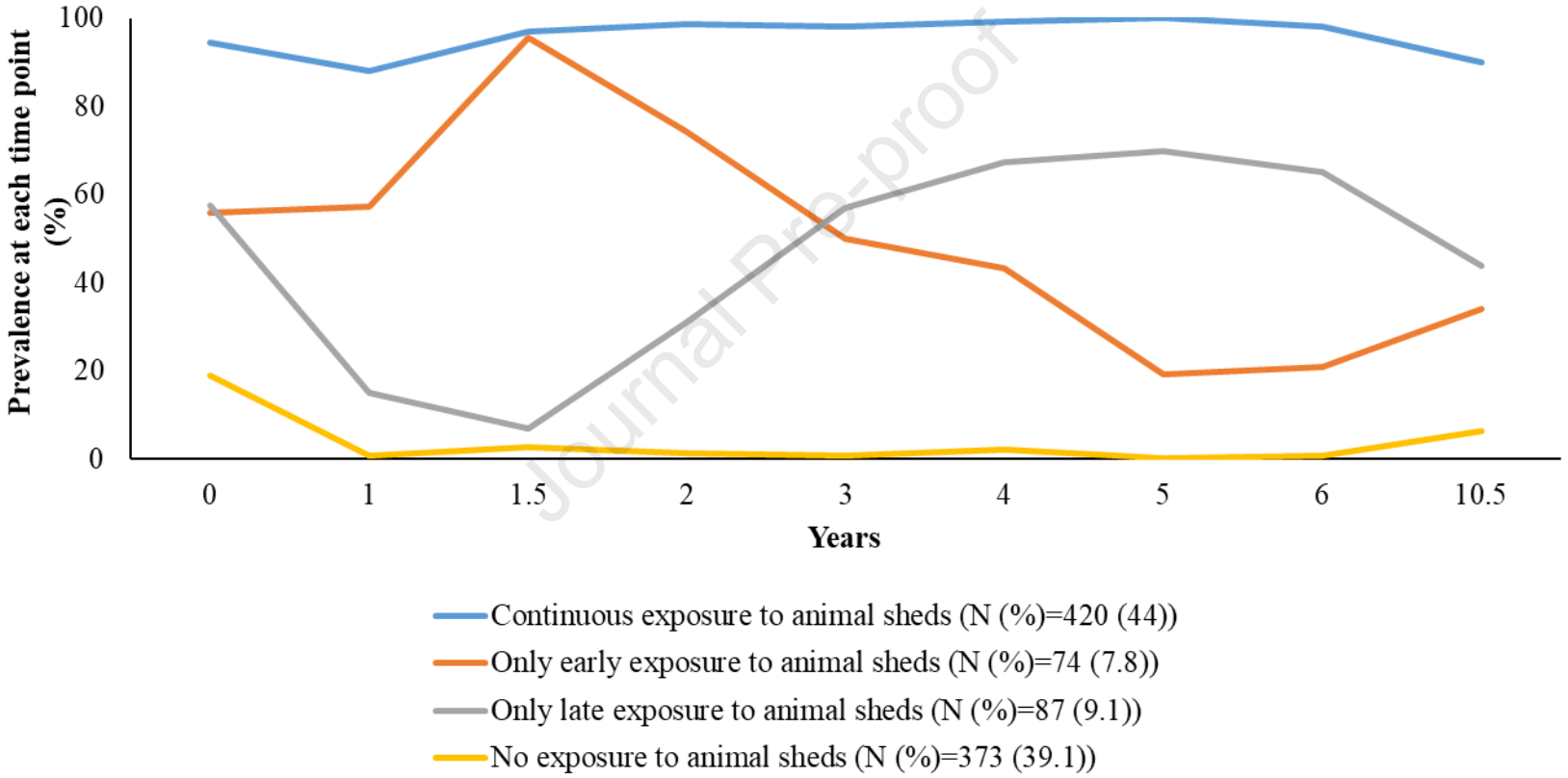
Parental atopy (yes)	416 (54.4)/765	72 (73.5)/98	344 (51.6)/667	*<0.0001*
Mode of delivery (normal)	624 (81.9)/762	82 (83.7)/98	542 (81.6)/664	*0.68*
Premature birth (yes)	11 (1.4)/769	1 (1.0)/99	10 (1.5)/670	*1.00*
Birth weight (kg) †	3.4±0.44 (N=605)	3.4±0.5 (N=82)	3.4±0.4 (N=523)	*0.81*
Breast feeding 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 $\geq 0.7 IU ml^{-1}$ 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 1.

a)



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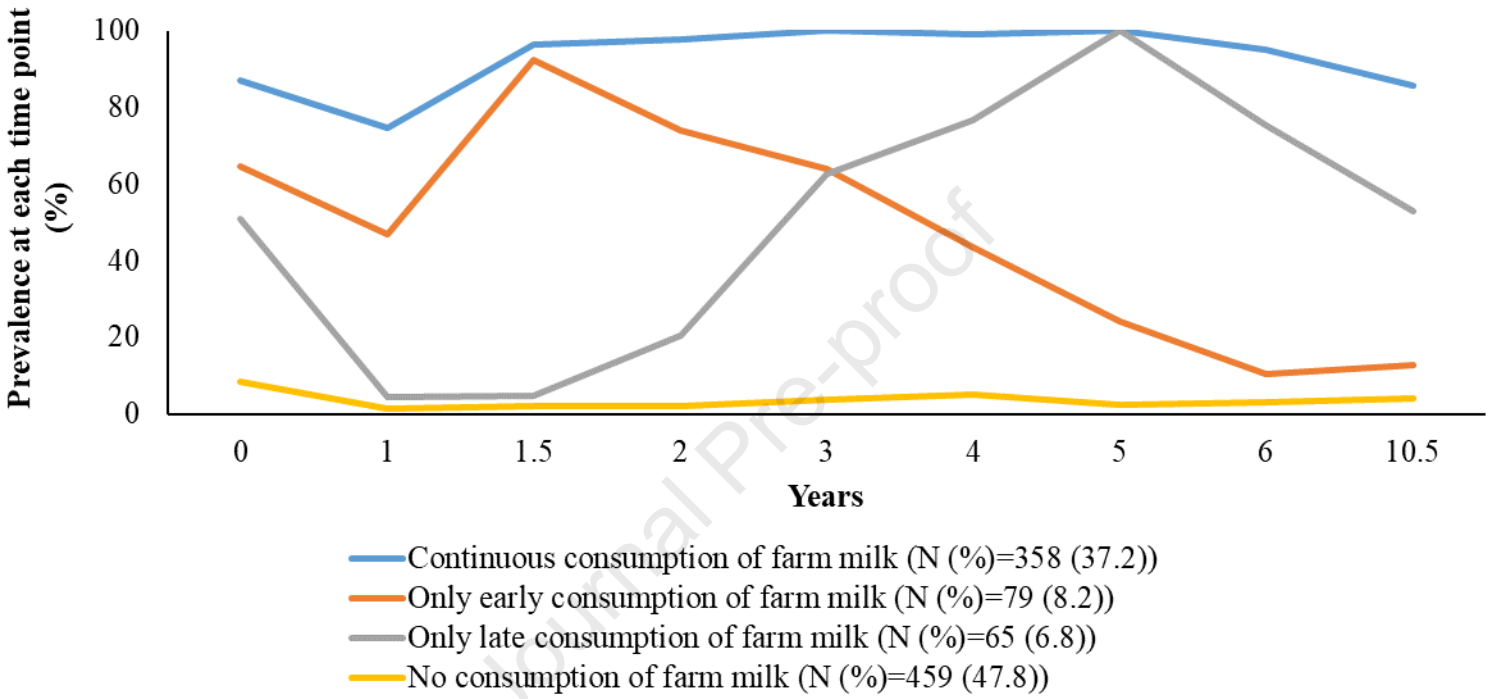
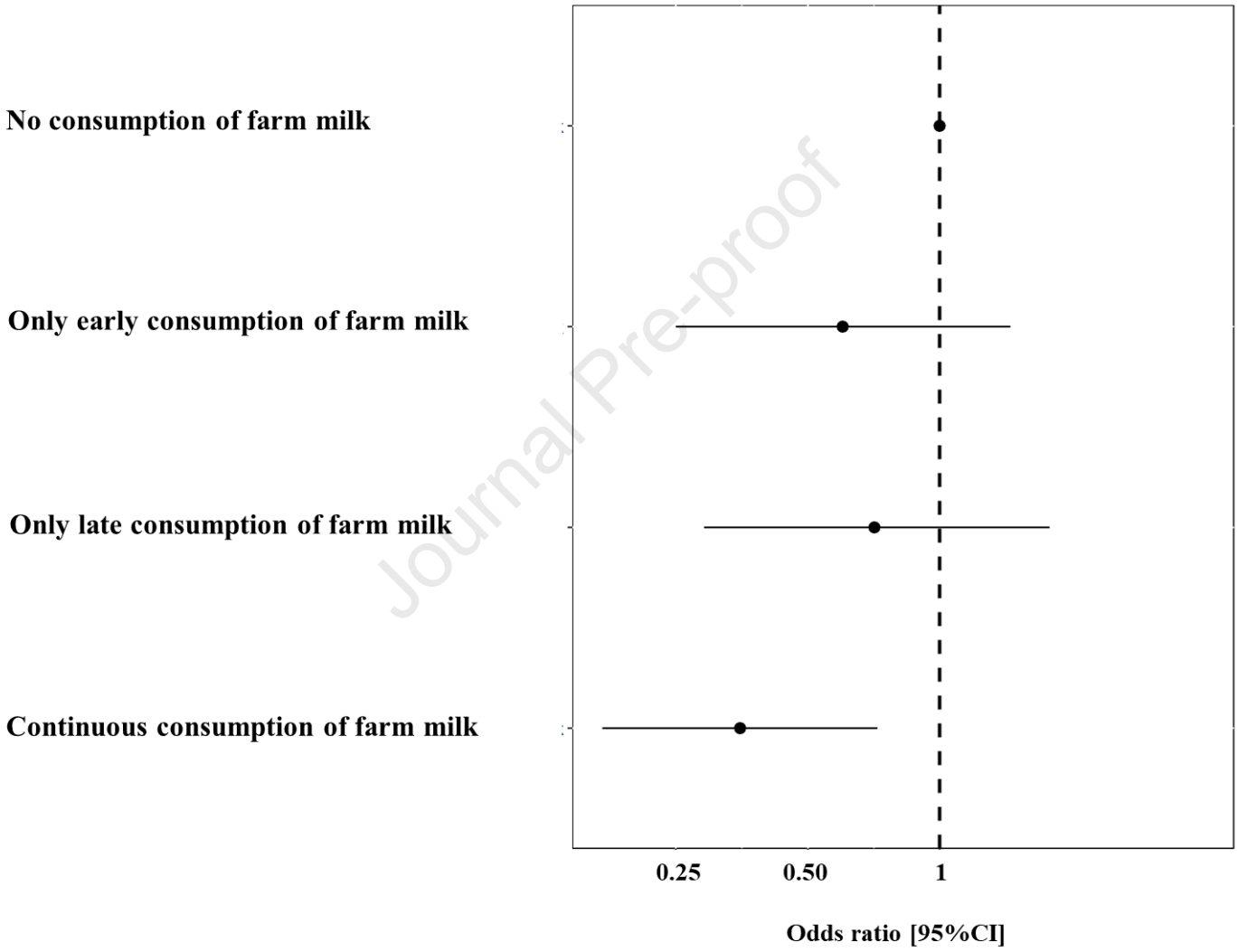


Figure 2



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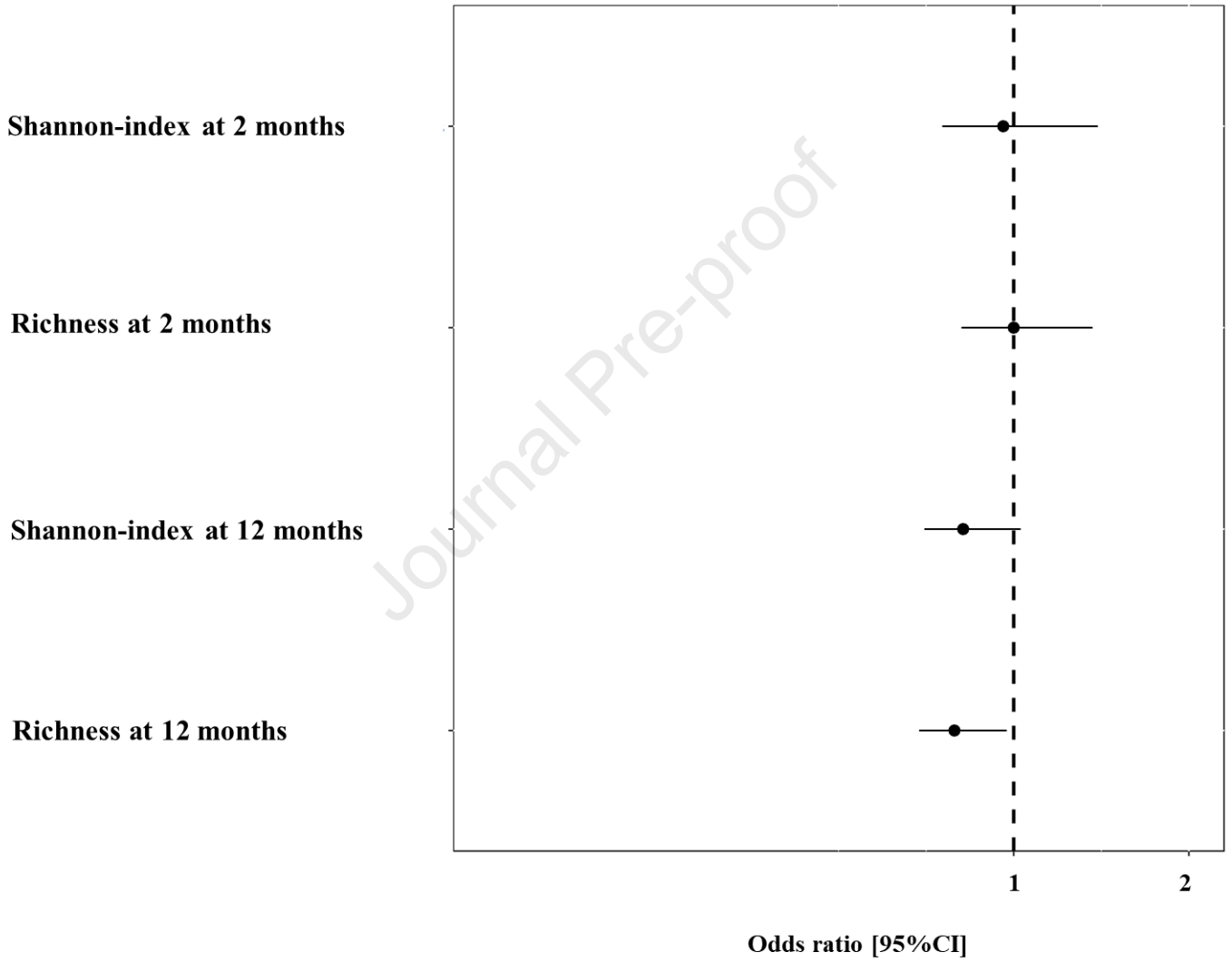

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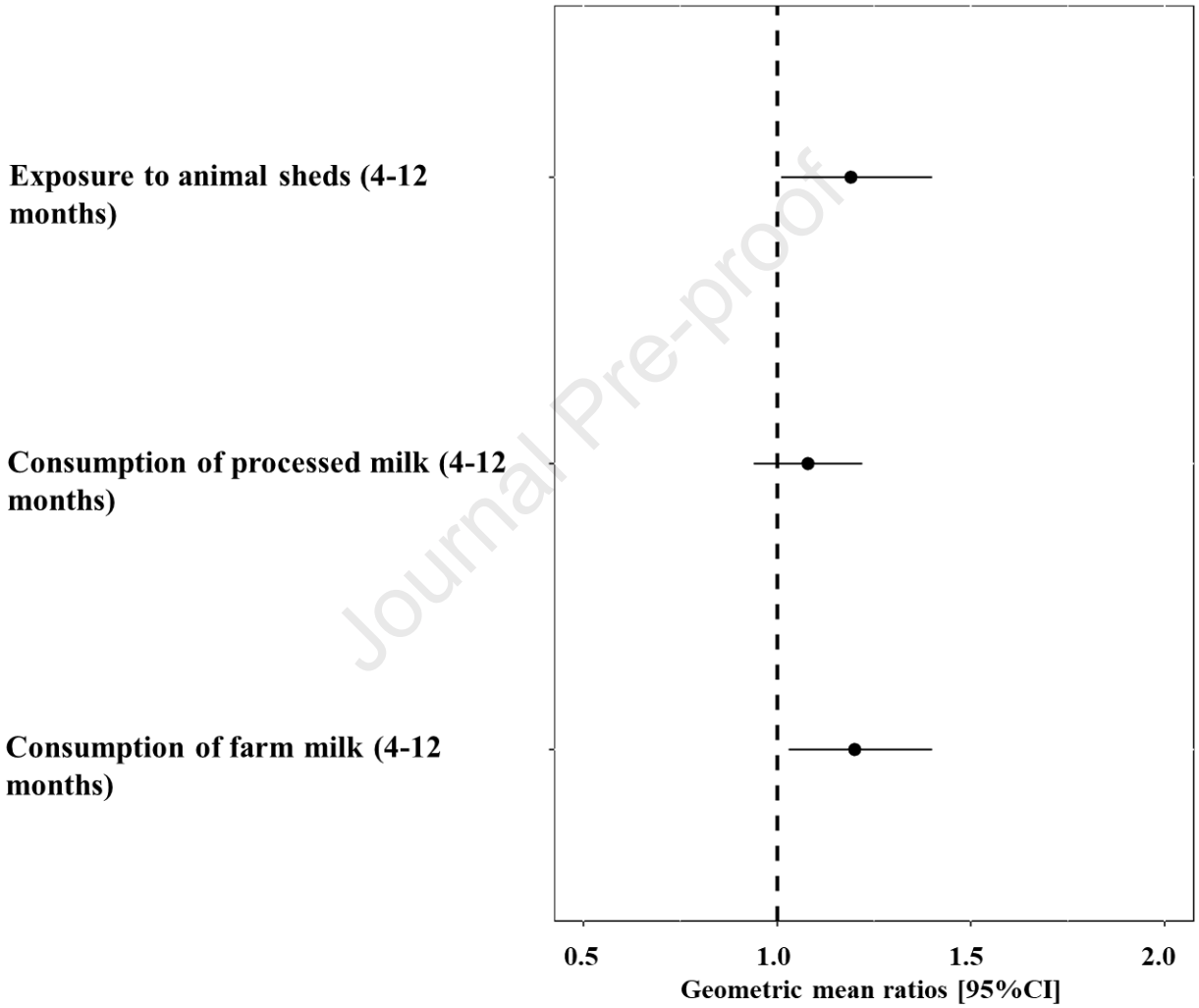

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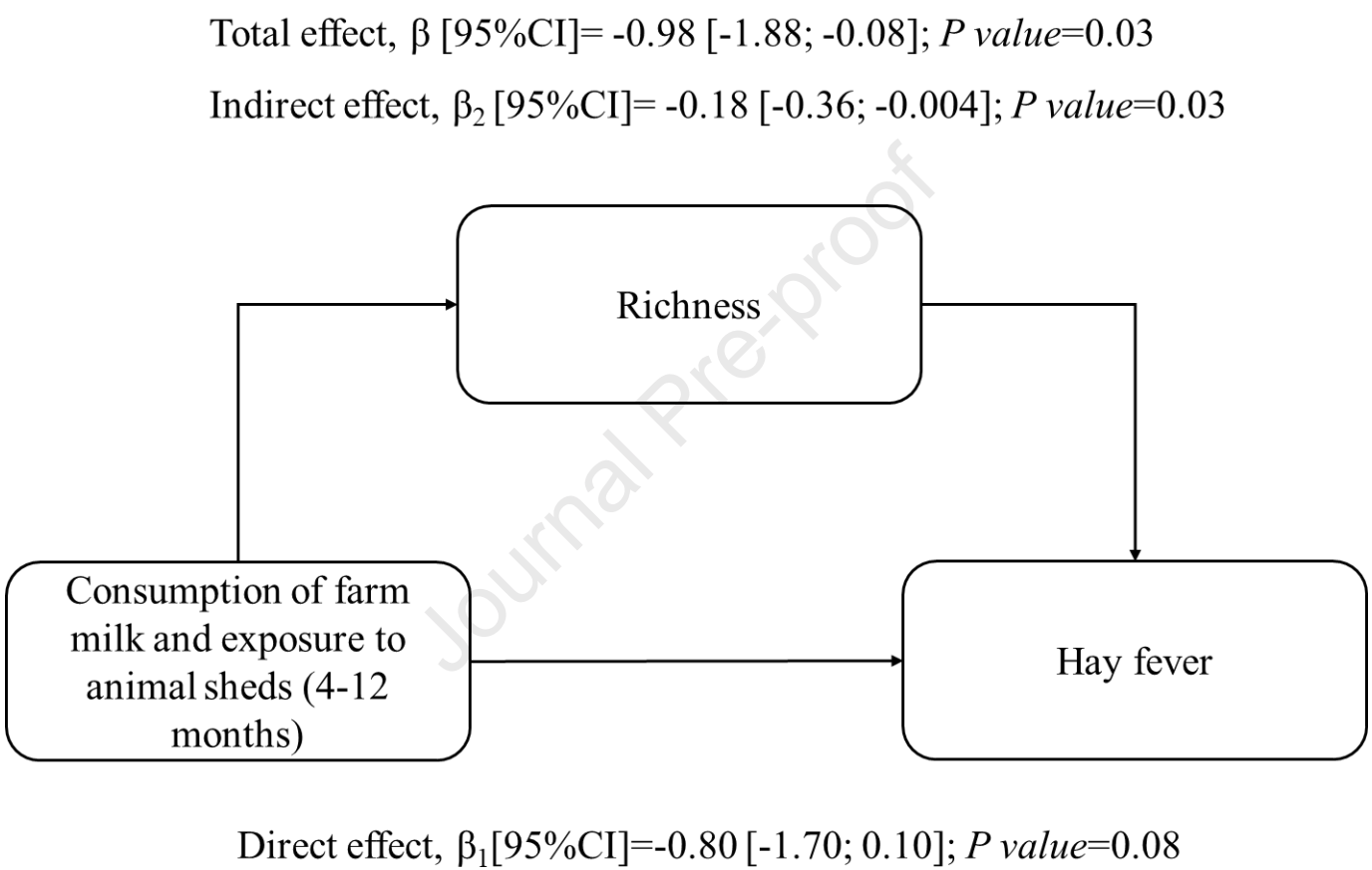
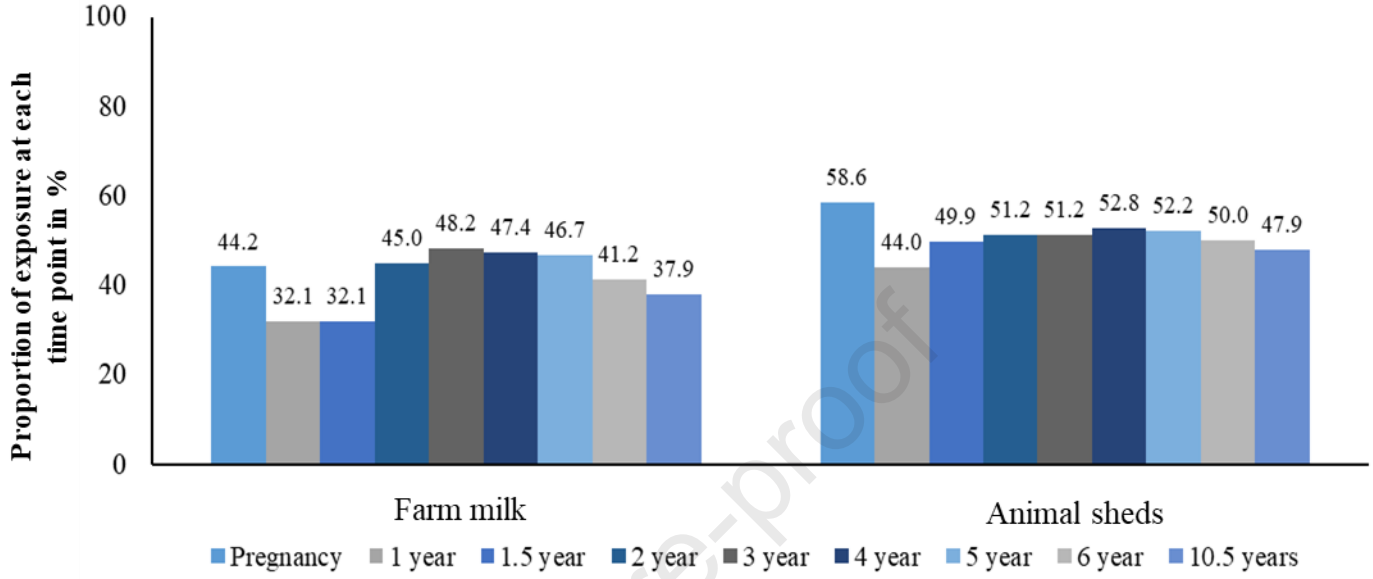


Figure E1.



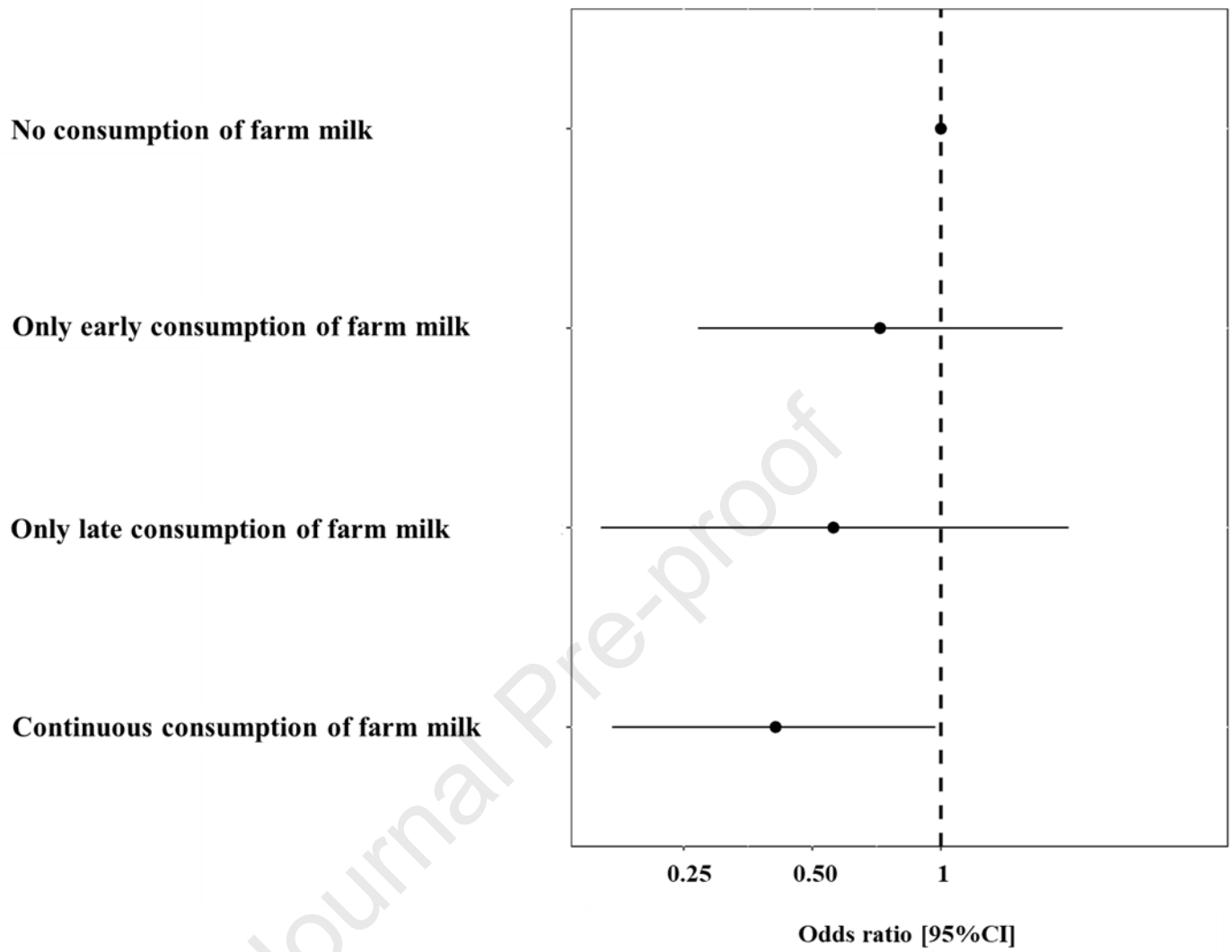
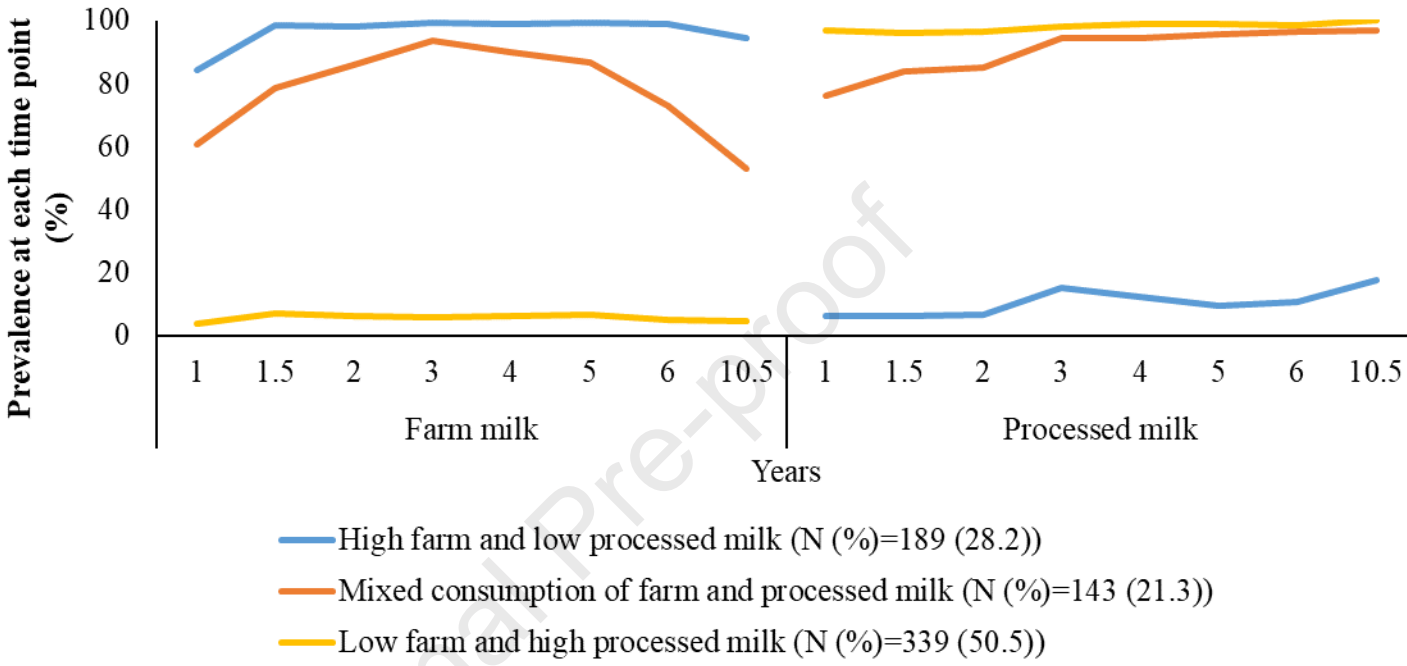


Figure E3.

(a)



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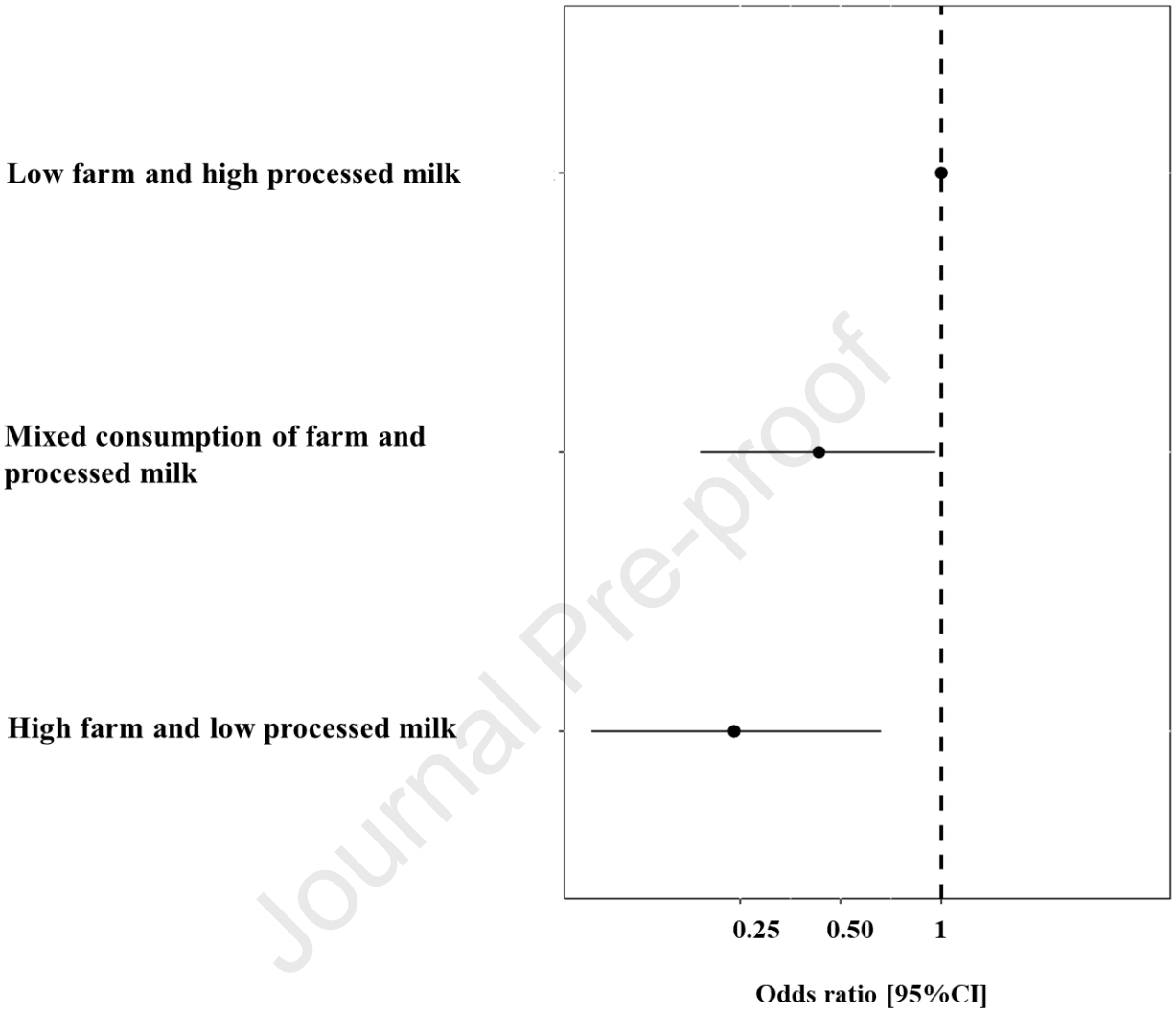

Figure E4.