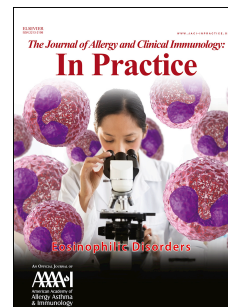


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Continuous rather than solely early farm exposure protect from hay fever development.

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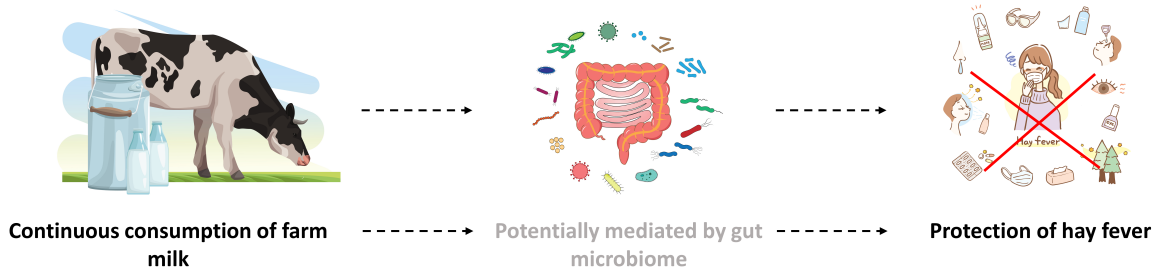
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1 **Continuous rather than solely early farm exposure protect from hay fever**
2 **development.**

3

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186 **Abstract**

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

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

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

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

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

206 **Highlight box:**

207 **1. What is already known about this topic?**

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

210 **2. What does this article add to our knowledge?**

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

214 **3. How does this study impact current management guidelines?**

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

218

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

220

221 **Abbreviations:**

222 PASTURE: Protection against Allergy-Study in Rural Environments

223 IgE: immunoglobulin E

224 SPT: skin prick test

- 225 RMLCA: repeated measure latent class analyses
- 226 q: quintile
- 227 aOR: adjusted odds ratio
- 228 95%CI: 95% confidence interval
- 229 IQR: interquartile range

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230 **Introduction**

231 Hay fever is the most common allergic disease worldwide with a prevalence between
232 20-30% (1). The high prevalence has a vast impact on several factors such as quality of
233 life and high healthcare costs (2, 3). Numerous epidemiological studies have shown the
234 protective effect of early life farm exposures and gut microbiome composition on
235 asthma, atopy, atopic sensitization, and hay fever (4-11), thus, proposing an important
236 'window of opportunity' for early life farm exposures and gut microbiome composition for
237 the protection of atopic diseases and asthma. However, it is unknown whether hay fever
238 with a peak incidence around late school age to adolescence is only determined very
239 early in life or whether later exposure before the onset of disease matters most.

240 The protective "farm-effect" has been attributed to two factors; consumption of
241 unprocessed cow's milk, subsequently termed 'farm milk' and exposure to animal sheds
242 (12-16). Hence, the aim of these analyses is to study the temporal pattern of these
243 protective exposures on hay fever development using the longitudinal data from the
244 PASTURE study. Furthermore, the role of the gut microbiome was investigated.

245 **Methods**

246 **Study design and population**

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

258 *Definitions of outcome:*

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

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

292 **Statistical analyses**

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

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

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

310 (continuous and quintiles), frequency of processed milk consumption, SCFAs (butyrate
311 score and propionate score) as well as gut microbiome's richness, and Shannon-index)
312 were assessed by logistic regression. We tested the differences in relative abundance
313 of most common single bacterial genera at 2 and 12 months with hay fever by Wilcoxon
314 test (10). The associations between gut microbiome richness and farm milk
315 consumption, processed milk consumption and exposure to animal sheds during infancy
316 was assessed by linear regression. The effect estimates are presented as adjusted
317 odds ratios (aORs) for logistic regression and geometric mean ratios (GMR; calculated
318 by exponentiation of the regression coefficients and their 95% confidence intervals
319 (95%CI)) for linear regression along with their respective 95%CI and a *P value* of 0.05
320 was considered significant. The above models were adjusted for centers and
321 confounders (growing up on a farm and parental asthma and/or atopy) associated with
322 hay fever and exposures in our study. No other confounders i.e. associated with both
323 outcome and exposures were found. We additionally calculated the Number Needed to
324 Treat (NNT), which is the effectiveness of a treatment on an outcome using an R-script
325 (22).

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

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

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334 **Results**

335 ***Characteristics of the study population***

336 At 10.5 year follow up 778 children participated in the PASTURE study and 769 have
337 data on hay fever. Comparing the baseline characteristics between included (N=769)
338 and excluded children (N=364) did not show any significant difference except for
339 maternal age at pregnancy, maternal smoking, parental education, and premature birth
340 (Table E1 Online Repository Text). Data on farm milk consumption and exposure to
341 animal sheds at least at one time point (from pregnancy, age of 12, 18 months, 2, 3, 4,
342 5, 6, and 10.5 years) was available for all these children. Of these, 769 children had
343 information on hay fever at 10.5 years of age. The proportion of children growing up on
344 a farm was 47.7%. Hay fever at the age of 10.5 years was reported in 12.9% children.
345 Of these, 28.9%, 36.7%, and 21.7% had asthma, eczema, and food allergy at age 10.5
346 years respectively (Table 1). Further, 86.8% were sensitized to inhalant allergens at age
347 10.5 years (Table 1). Figure E1 (Online Repository Text) shows the proportion of
348 children who were consuming farm milk or were exposed to animal sheds at each time
349 point. The consumption of farm milk by children increased from the age of 1 to 3 years
350 and gradually decreased after age 4 years. Similarly, exposure to animal sheds also
351 increased from the age of 1 to 4 years and slightly decreased after age 5 years.

352 ***Temporal pattern of the farm-related exposures on hay fever***

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

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

363 We then analyzed the temporal pattern of consumption of farm milk in similar categories
364 'continuous consumption of farm milk', 'only early consumption of farm milk', 'only late
365 consumption of farm milk' and 'no consumption of farm milk' (Figure 1(b)). The
366 strongest inverse association was found for the 'continuous consumption of farm milk'
367 as compared to 'no consumption of farm milk' (0.35 [0.17; 0.72], 0.004) exposure class
368 (Figure 2 and Table E3 Online Repository Text). In contrast, 'only early consumption of
369 farm milk' showed no significant effect on hay fever. The inverse association of
370 'continuous consumption of farm milk' compared to 'no consumption of farm milk' was
371 still observed when using the stringent definition of hay fever (0.41 [0.17; 0.97], 0.04)
372 (Figure E2 Online Repository Text) or incident hay fever at age 10.5 years (0.39 [0.15;
373 0.99], 0.05, data not shown). Since confounding by reverse causation might have
374 biased our findings, we ran a sensitivity analysis in the subgroup of children without a
375 family history of parental asthma and/or atopy and excluded children avoiding milk or
376 milk products at the age 1–6 years. This did not change the inverse association with hay
377 fever (0.21 [0.06; 0.78], 0.02, data not shown).

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

385 We further investigated if consumption of processed milk shows similar effects as
386 consumption of farm milk (Figure E3(a) Online Repository Text). Consumption of 'high
387 farm and low processed milk' was inversely associated with hay fever (0.24 [0.09; 0.66],
388 0.006), however, the consumption of processed milk attenuated the farm milk effect
389 when both farm milk and processed milk were consumed ('mixed consumption of farm
390 and processed milk' (0.43 [0.19; 0.96], 0.04) (Figure E3(b) and Table E3 Online
391 Repository Text). Furthermore, daily consumption of shop milk at the age of 10.5 years
392 showed association in positive direction with hay fever (Figure E4 Online Repository
393 Text).

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

397 ***Early life effect of gut microbiome on hay fever***

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

401 We did not find any significant differences in relative abundance of most common
402 bacterial genera at 2 and 12 months with subsequent hay fever at 10.5 year (data not
403 shown). Also, richness and Shannon-index of bacteria at 2 months were not associated
404 with hay fever at 10.5 years (Figure 3). However, the bacterial richness of the gut
405 microbiome at 12 months was inversely associated with hay fever (aOR [95%CI], *P*
406 *value*: 0.66 [0.46; 0.96], 0.03, Figure 3). Shannon-index at 12 months also showed an
407 inverse non-significant trend for hay fever (0.71 [0.49; 1.04], 0.08, Figure 3). The SCFAs
408 butyrate (1.00 [0.92; 1.09], 0.99) and propionate scores (0.97 [0.90; 1.05], 0.50) were in
409 turn not associated with hay fever (data not shown). We reasoned that consumption of
410 milk and exposure to animal sheds may shape the gut microbiome, in particular its
411 richness. Consumption of farm milk (aGMR [95%CI]: 1.20 [1.03; 1.40], *P value*=0.02)
412 and exposure to animal sheds (aGMR [95%CI]: 1.19 [1.01; 1.40], *P value*=0.04) in the
413 first year of life increased gut microbiome richness (Figure 4). In turn, no association
414 was observed for consumption of processed milk (Figure 4). Since both, farm milk
415 consumption and exposure to animal sheds during infancy (4-12 months) showed
416 significant associations with gut microbiome richness at 12 months, we performed a
417 mediation analysis including unexposed and children exposed to both in infancy. The
418 mediation analysis revealed that part (18.4%) of the total protective effect of farm milk
419 consumption and exposure to animal sheds in the first year of life on hay fever was
420 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.

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423 Discussion

424 In the PASTURE birth cohort, the continuous consumption of farm milk throughout age
425 10.5 years, but neither the only early nor the only late exposure alone was significantly
426 associated with reduced risk of hay fever at age 10.5 years. In contrast, exposure to
427 animal sheds only exerted a trend towards protection early in life. Both exposures, farm
428 milk and animal sheds, early in life increased gut microbiome richness at age 12
429 months, which partly explained the protective effect of these exposures on hay fever.

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

439 In contrast, gut microbiome richness at the age of 1 year was inversely associated with
440 hay fever at age 10.5 years. We have previously shown in the PASTURE cohort in
441 agreement with others that the compositional structure of the gut microbiome undergoes
442 very significant changes from early age when most infants are breastfed to age 12
443 months when most foods have been introduced into a child's diet (10, 11).
444 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.

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

477 Exposure to animal sheds during early years showed an inverse, albeit non-significant
478 effect on hay fever. This is in contrast to previous farm studies showing stronger effects
479 (12, 32). The discrepancy might be attributable to important differences in the definition
480 of exposure to animal sheds used in the PASTURE study, which only assessed
481 exposure to any animal sheds without differentiating between cows, pigs, sheep and
482 horses. The nature of animal exposure may however matter. While exposure to cow
483 sheds showed a significant protective effect on hay fever and asthma (12), sheep sheds
484 and keeping of hares and rabbits were risk factors for wheezing and asthma
485 respectively in the PARSIFAL farm study (33).

486 The main strength of this study is its longitudinal design, which enabled us to assess the
487 exposures at several time points before the assessment of the outcome. Excluding
488 children with parental asthma and/or atopy and who were avoiding milk or milk products

489 showed similar inverse associations with hay fever consequently arguing against
490 confounding by reverse causation. An elevated risk of diarrhea and farm milk
491 consumption at 10.5 years was not observed (data not shown). The results of the
492 present study show protective association of continuous consumption farm milk on hay
493 fever. However, one of the potential caveats of the observation study is finding
494 causality. Hence, the Milk Against Respiratory Tract Infections and Asthma (MARTHA)
495 an ongoing interventional trial is being carried out to evaluate the preventive effect of
496 minimally treated, i.e. only pasteurized and thus microbiologically safe cow's milk on
497 upper respiratory tract infections and allergy (34). Further, the NNT in our study was 7,
498 however, this study is not a randomized placebo-controlled double-blind trial and thus
499 numbers must be taken with some caution. One of the drawbacks of the study is the
500 missing data on hay fever at 10.5 years. However, comparing the baseline
501 characteristics between included and excluded children did not show any significant
502 difference except for maternal age at pregnancy, maternal smoking, parental education,
503 and premature birth. However, adjusting for these variables did not change the results
504 (data not shown). Another drawback is the small number in the "only early" and "only
505 late" exposure groups that shows protective non-statistical significant effect on hay
506 fever. However, using the RMLCA approach our study could identify these small groups
507 manifesting that these types of habits i.e. farm milk consumption or exposure to animal
508 sheds do exist. We performed a posthoc power calculation using SAS and considering
509 $\alpha=0.05$ (two-sided). For our sample size of 650, i.e. in the exposure groups 'continuous
510 consumption of farm milk' and 'no consumption of farm milk' the power of study is over
511 80% assuming the response probabilities ranging from 0.02-0.18 for having hay fever in

512 children who consume farm milk and unadjusted OR of 0.24. Thus, our study was well
513 powered to detect a relatively strong effect of farm milk consumption on hay fever.
514 In summary, the results of the present study demonstrate that continuous exposure of
515 the main determinant, i.e. farm milk consumption but neither only early nor only late
516 exposure alone conferred protection from hay fever development. The early
517 compositional structure of the gut microbiome at age 1 year, but not age 2 month, did
518 however in part mediate this protective effect. One might speculate that continuous
519 consumption of unprocessed cow's milk may also increase gut microbiome richness at
520 later ages, but we do not have data to support this notion. Overall, the findings
521 presented herein do not support the notion of an early-determined trajectory where only
522 early exposures in the first months of life would govern later outcomes. These results
523 emphasize the preventive potential of continuously drinking unprocessed farm milk for
524 hay fever protection. However, the risks associated with raw cow's milk consumption
525 prohibit its recommendation for daily life. The results of the MARTHA trial however will
526 shed light on potential side effects (34). Further clinical trials based on the present
527 results are warranted.

528

529

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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,

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

647 **Figure 5.** Mediation analysis.

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

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

Characteristic	All	Hay fever	No hay fever	<i>P value</i>
	(N=769)	(N=99 (12.9%))	(N=670 (87.1%))	
	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

654

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656

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 χ^2 or Fischer's Exact test for categorical variables and Mann Whitney U test for continuous variables.

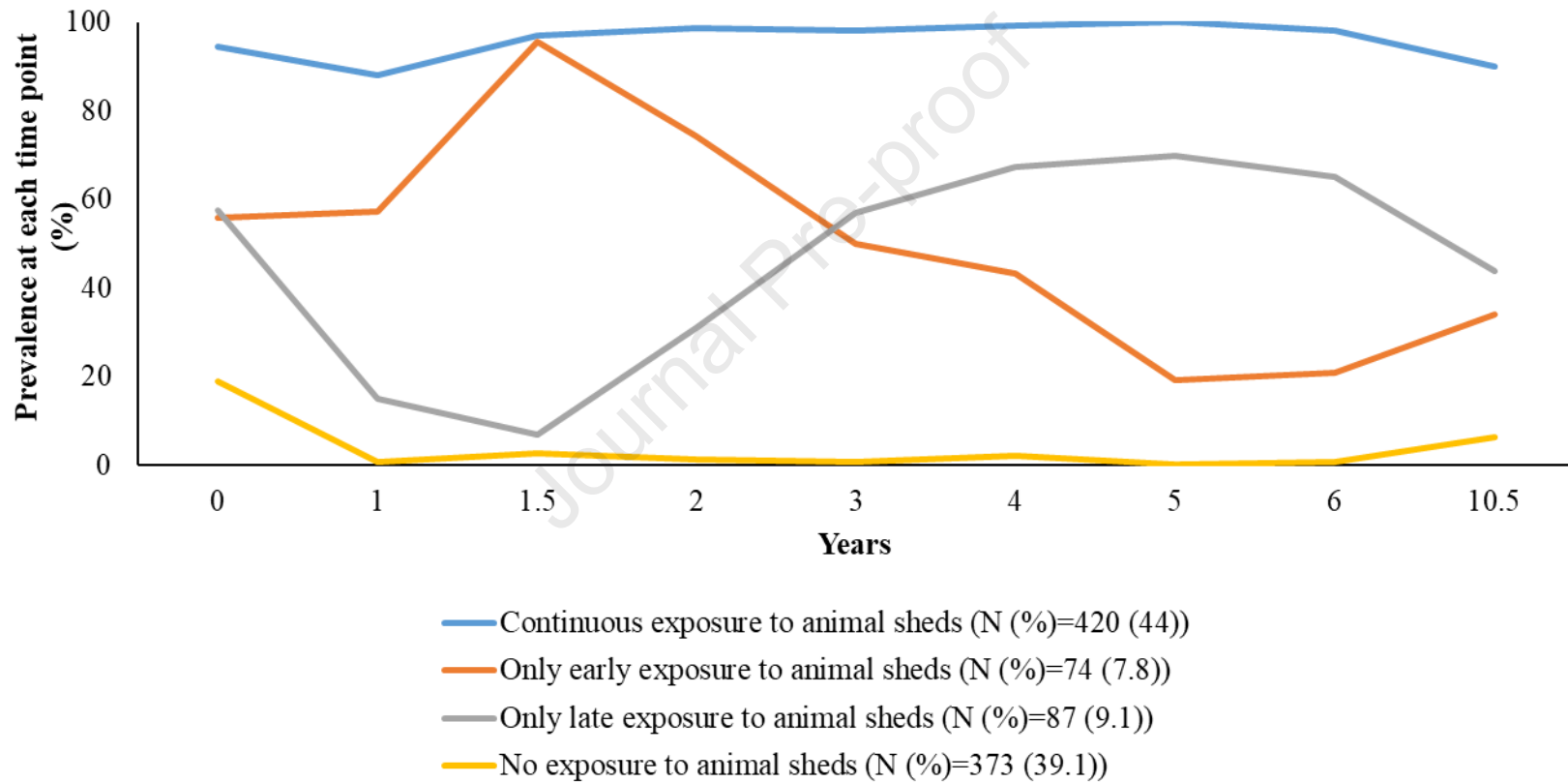
657 Farm child was defined as “Children of mothers living on family-run livestock farms were assigned to the farm group. The non-farm group included children of
658 mothers from the same rural areas but not living on a farm”. Exposure to pets at the age of 2 months (cats and dogs) was defined by asking “if you have cats?”,
659 “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
660 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,
661 “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
662 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
663 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
664 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
665 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
666 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
667 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,
668 plantain, mugwort, alternaria, grass, rye, *Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, cat, dog, or horse at levels $\geq 0.7 \text{IUml}^{-1}$ or SPT (birch,
669 grass, alternaria, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, or dog) $\geq 3 \text{mm}$. Serum specific IgE and SPT was not measured in the
670 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
671 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.

672

673

Figure 1.

a)



b)

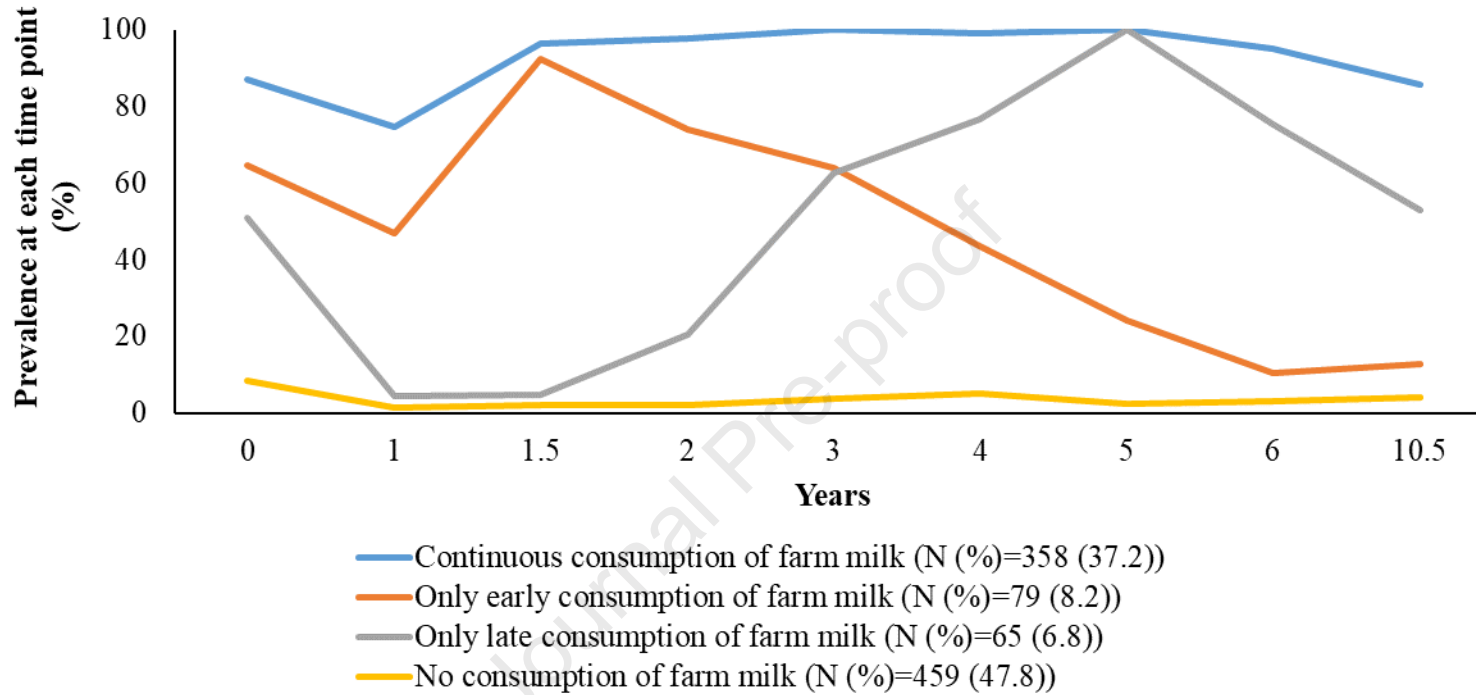


Figure 2

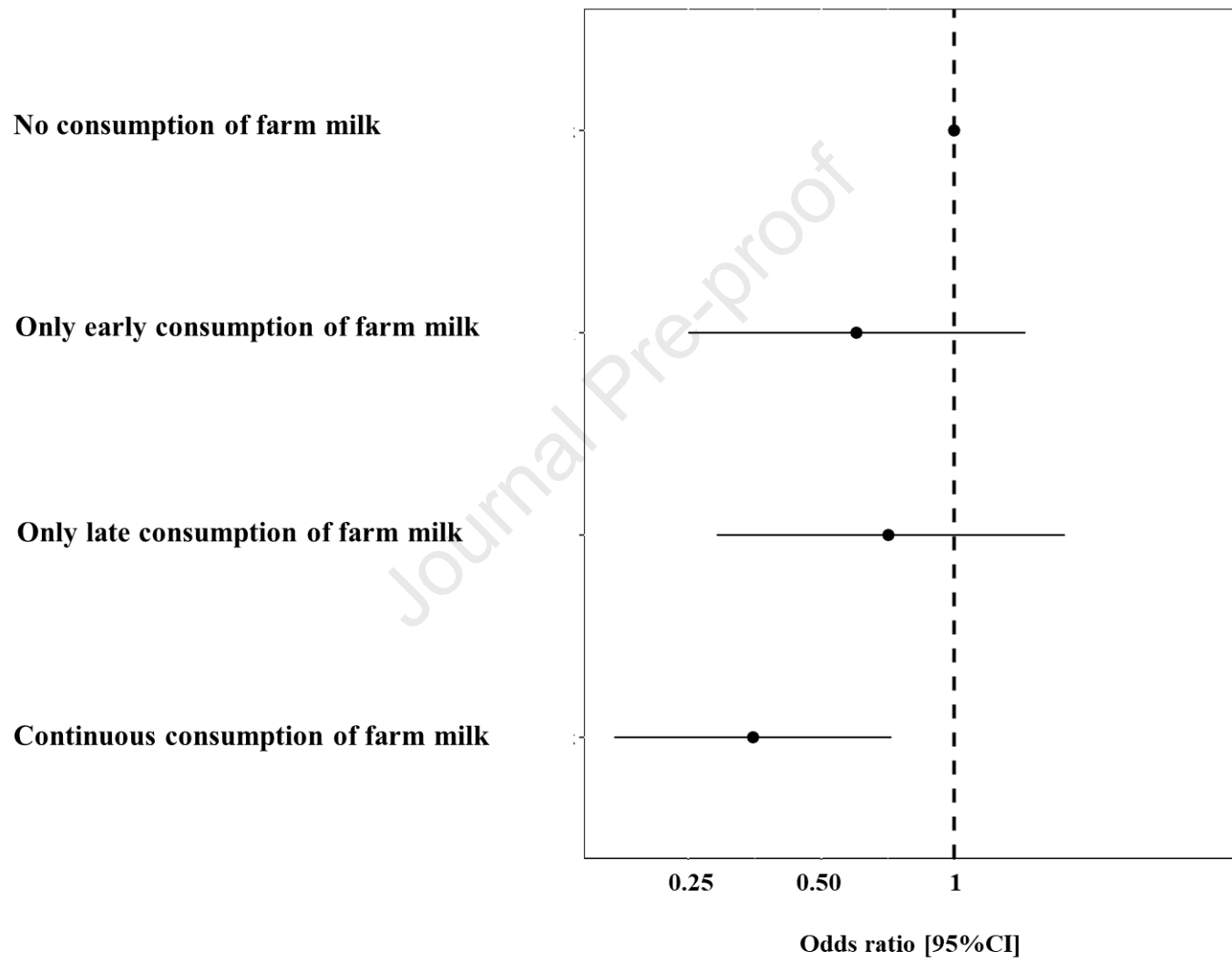


Figure 3

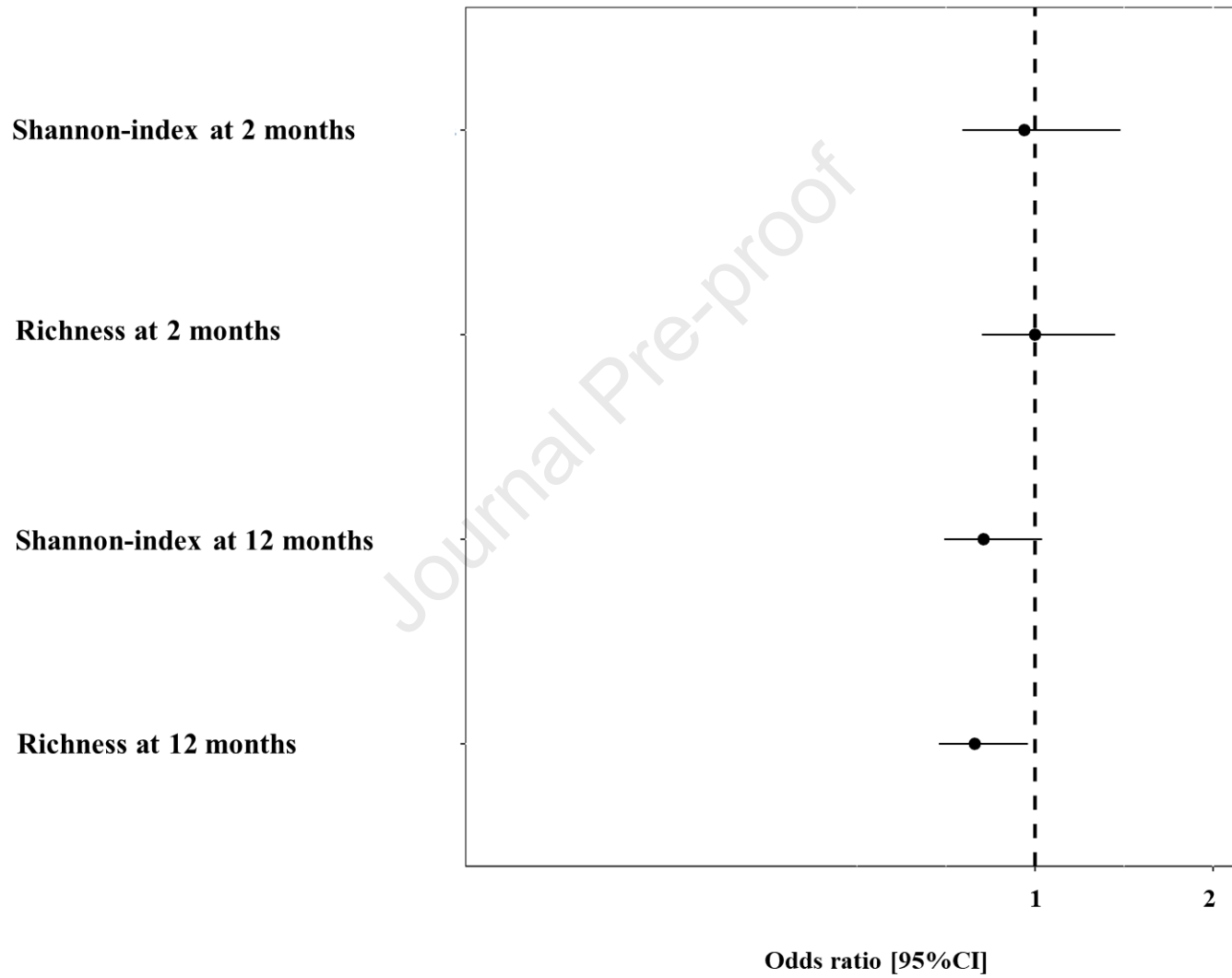


Figure 4.

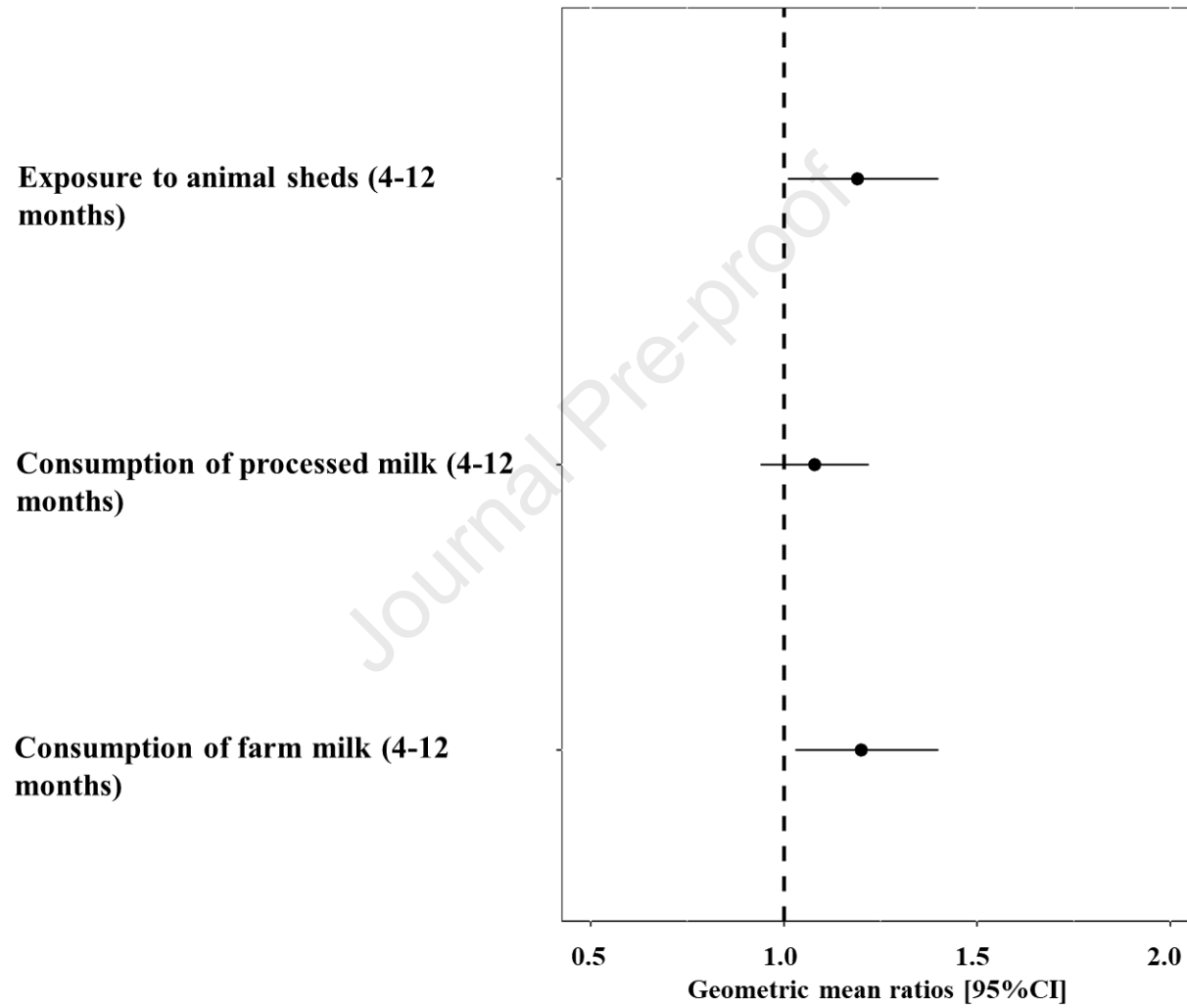
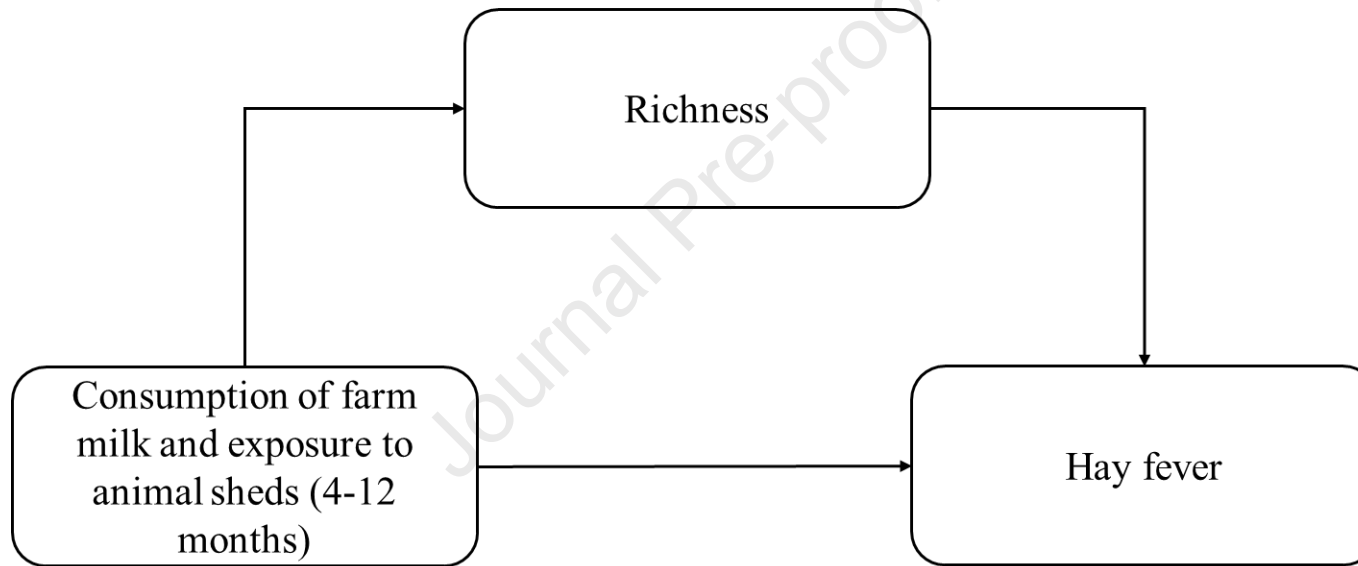


Figure 5.

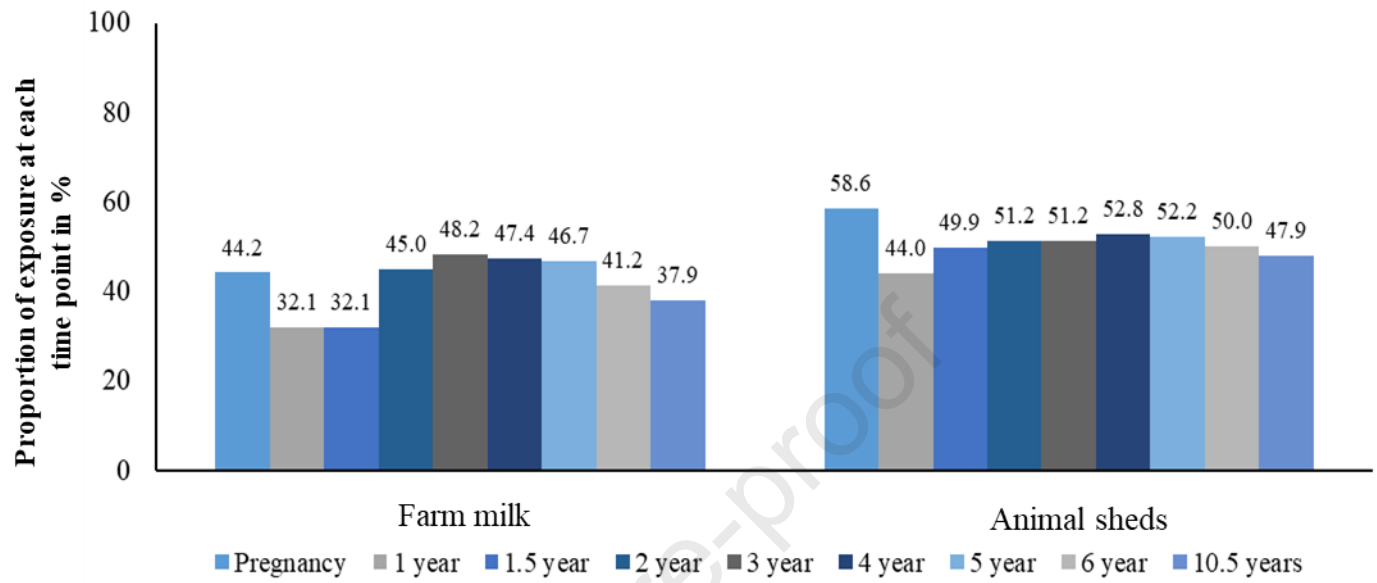
Total effect, β [95%CI]= -0.98 [-1.88; -0.08]; *P value*=0.03

Indirect effect, β_2 [95%CI]= -0.18 [-0.36; -0.004]; *P value*=0.03



Direct effect, β_1 [95%CI]= -0.80 [-1.70; 0.10]; *P value*=0.08

Figure E1.



No consumption of farm milk

Only early consumption of farm milk

Only late consumption of farm milk

Continuous consumption of farm milk

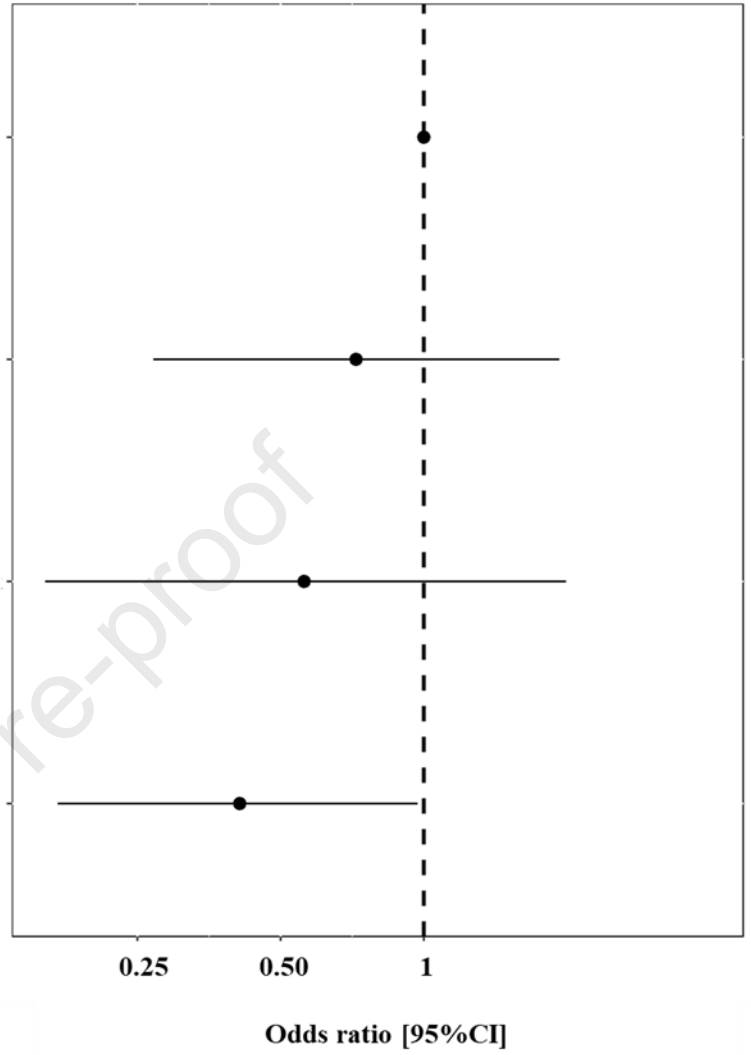
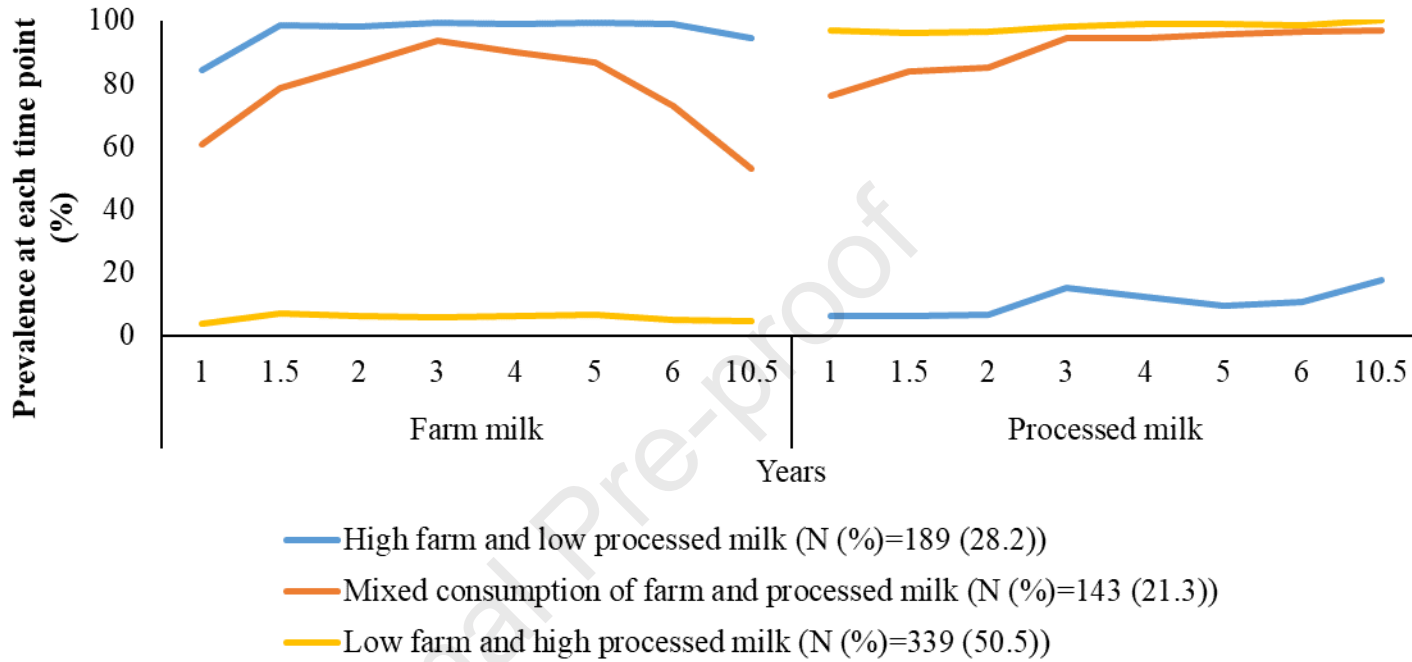


Figure E3.

(a)



(b)

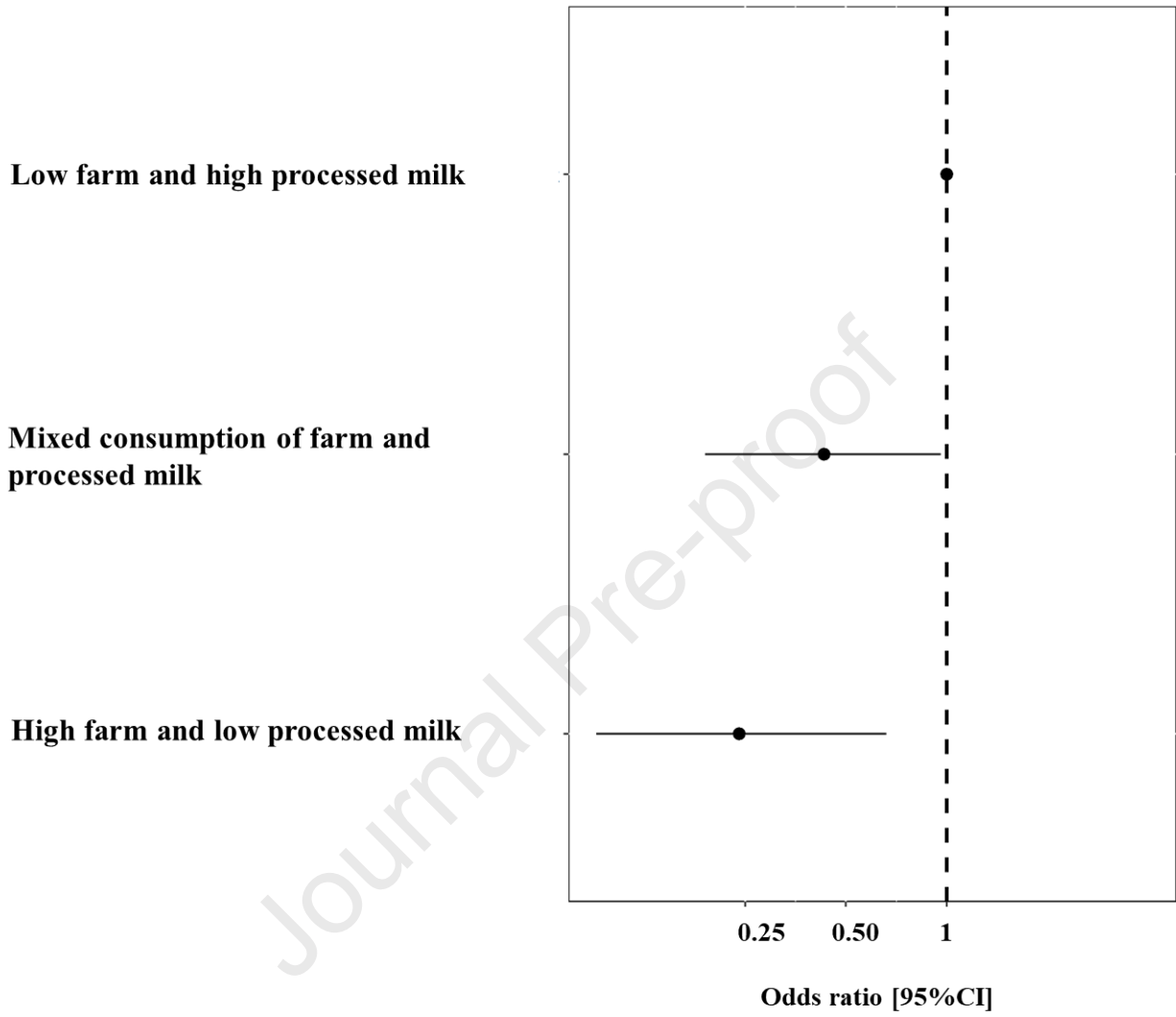


Figure E4.

