Investigating the Influence of Pesticide Exposure on the Human Gut Microbiome – MSc Epidemiology Writing Assignment, Research Proposal

Master's Writing Assignment
MSc Epidemiology

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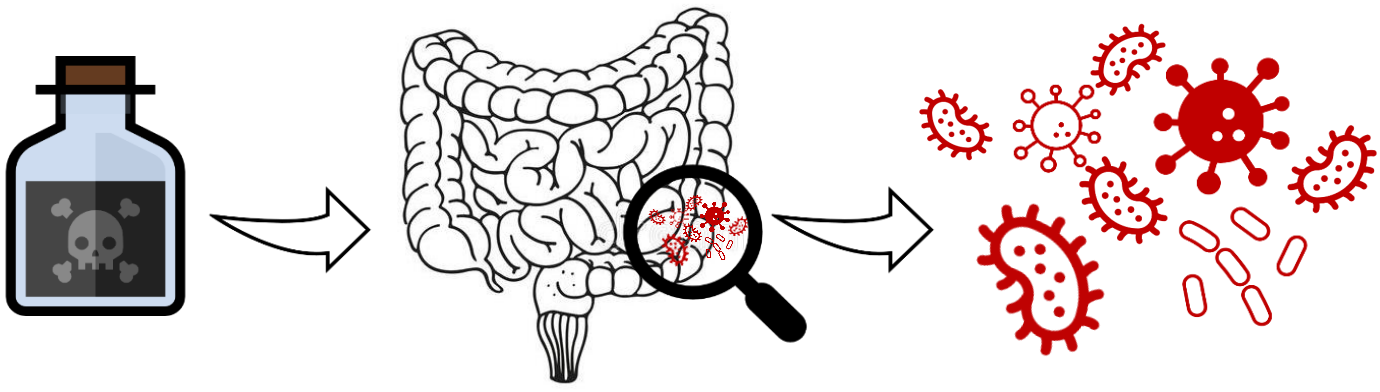
Study name: **Gu**t **M**icrobiome **P**esticide **Ex**posure **(GuMPEx)**



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1. Abstract

Background: Pesticides are widely used in the agricultural industry and can cause unintentional harm to non-target organisms, including humans. The public health effects of exposure to pesticides have been widely examined, however impacts on the human gut microbiome have been rarely investigated. Dysbiosis of the gut microbiota has been linked with many disease states and could have critical associations with the adverse health effects observed following pesticide exposure. Experimental animal studies have shown significant alterations of the microbial structure following both short-term and long-term exposure to pesticides but further studies are required to clarify the microbial effects in humans.

Objectives: Our primary aim is to determine whether personal exposure measurements reflecting short- and medium-term pesticide exposure are linked with structural alterations of the human gut microbiome. Additionally, we aim to investigate the impacts of long-term pesticide exposure on microbial structure by comparing the gut microbiomes of organic and non-organic farmers.

Methods: Short-term pesticide exposure will be defined and quantified using measured urine concentrations of five specific pesticide metabolites. Proxies of medium-term pesticide exposure include analysis of compounds adsorbed by silicone wristbands worn by participants and analysis of participant hair samples. Non-organic farmers will represent individuals with long-term pesticide exposure. To explore the impacts of short-, medium- and long-term pesticide exposures on the gut microbial structure, we will inspect different characteristics of the microbiome. Alpha and beta diversity will be explored in relation to pesticide exposures and we will also determine whether there are specific differentially abundant bacterial species in relation to pesticide exposure using differential abundance analysis.

Expectations and applicability of results: As a result of pesticide exposure, we expect to find significant alterations in the gut microbial structure. By elucidating possible pesticide-induced gut microbial alterations, we hope to determine whether the gut microbiome should be a target of interest to investigate in future safety assessments of pesticides. By investigating the effects of different exposure durations, we will be able to identify individuals at greatest risk, which may drive the development of additional exposure prevention strategies.

Keywords: Pesticides, gut microbiome, occupational exposure, organic farming.

2. Lay summary

Pesticides are substances that are designed to control undesired plants or animals, and are commonly used in agriculture to increase crop yields. There are several types of pesticides used on agricultural fields including herbicides (to control plants), insecticides (to control insects) and fungicides (to control fungi). In the Netherlands alone, five million kilograms of pesticides were used on crops in 2020 and of all grown crops, flower bulb cultivation constitutes the largest use in the country (Statistics Netherlands, 2022).

There are a wide range of health effects that have been associated with pesticide exposure. These include both short-term effects such as rashes, blisters and nausea, but also chronic effects such as cancers, diabetes and endocrine disruption. Due to their designed purpose to kill, it is likely that these substances will disrupt the bacteria present within the human body. The human body is filled with bacteria, many of which are harmless and help to maintain good health. In fact, the human body contains more bacterial than human cells (Sender et al., 2016). Across the whole body, the gut contains the most bacteria and is highly diverse, with over 1,000 bacterial species present (Human Microbiome Project Consortium, 2014). The collection of bacteria within the gut is called the gut microbiota and the gut microbiome refers to the collection of genomes from all the microorganisms within the gut.

Alterations in the structure of the gut microbiome have been associated with many disease states such as metabolic, autoimmune, cardiovascular and respiratory diseases (Vijay & Valdes, 2022). It is possible that

the adverse health effects observed as a result of pesticide exposure are modulated by changes in the gut microbiome. There have been multiple animal studies investigating whether the gut microbiome is altered in response to controlled exposures to pesticides and these studies have found significant alterations of gut microbial structure following pesticide exposure. In addition, the human gut microbiome has been artificially simulated using a multiple-chamber reactor (van de Wiele et al., n.d.), and used to investigate impacts of pesticide exposure. Significant changes in abundances of specific bacterial species residing in the gut simulator were observed following pesticide exposure (Joly et al., 2013; Reygner et al., 2016). Although animal studies and simulations provide useful estimates of the possible effects on the human gut microbiome, we cannot in certainty extrapolate these effects directly to the human gut microbiome. As yet, no studies have directly investigated the impacts of pesticide exposure on the microbial structure of the human gut. Therefore, we aim to explore this uncharted territory by designing a study to quantify changes in the human gut microbiome following occupational exposure to pesticide mixtures from agricultural use.

The study involves flower bulb farmers in the Netherlands, including both organic (non-pesticide using) and non-organic (pesticide-using) farmers. We will also involve a group of non-farmers within the study so that results can be generalised to a larger population. We will take faecal samples from all participants and characterise the microbiome of these as this is a good representative of the gut microbiome (Tang et al., 2020). To assess exposure to pesticides, concentrations of pesticides within urine samples, wristbands and hair from participants will be quantified.

3. Background and relevance

Pesticides are broad group of chemical substances that are used to control undesired insects, fungi and plants from agricultural crops and are widely used in modern agriculture as a highly effective means of increasing agricultural production. Approximately two million tonnes of pesticides are used annually worldwide (Sharma et al., 2019), and usage worldwide continues to rise. Despite their benefits, pesticides have far reaching impacts, with toxic effects on many non-target organisms. Their usage pollutes the soil, water and air in addition to having wider knock-on effects on human and ecosystem health. Global concerns surrounding the usage of pesticides have been rising, and in 2001, the Stockholm Convention on Persistent Organic Pollutants was signed with the aim to eliminate or restrict the production and use of these substances which include pesticides.

Exposure to pesticides poses a large public health threat, with both acute and chronic health effects. Long-term exposure to pesticides has been associated with a plethora of diseases including cancer (Van Maele-Fabry et al., 2010), asthma (Rodrigues et al., 2022) and congenital abnormalities (Asghar et al., 2016). Although some disease-causing mechanisms of pesticides have been identified, the link between pesticide exposure and many of the adverse health effects observed have not been elucidated. It could be that dysbiosis of the gut microbiome plays a role in these causal pathways. The gut microbiome refers to the combined genetic material of the microorganisms found within the gut. It is known that the gut microbiome is one of the key contributors in the regulation of host health, and it has been shown that dysbiosis is associated with many diseases such as obesity (Fetissov, 2017), type 2 diabetes (Karlsson et al., 2013; J. Wang et al., 2012) and colorectal cancer (Tilg et al., 2018). The gut has the highest microbial mass in the whole body and has been the most comprehensively studied of all microbiomes (Lloyd-Price et al., 2017).

In general, the gut microbiome is relatively stable but several factors are known to be associated with changes in the gut microbial structure including age, genetics, diet, pregnancy, socioeconomic status and environmental exposures (National Academies of Sciences Engineering and Medicine, 2018). Increasing attention is being paid to the environmental exposure effects on the microbiome and several exposures have been identified as determinants of the microbiome including antibiotic use (Ramirez et al., 2020), air pollution (Fouladi et al., 2020) and heavy metals (Lu et al., 2014). These results provide stimuli for further

studies to explore environmental exposure effects on the microbiome. There is an increasing awareness that chemicals such as pesticides may alter the microbial composition within the human body (National Academies of Sciences Engineering and Medicine, 2018). Studies have shown that exposing mice and zebrafish to chlorpyrifos, a ubiquitous pesticide, causes significant dysbiosis of the gut microbiome (X. Wang et al., 2019; Yan et al., 2020; Zhao et al., 2016). Simulation studies using the "Simulator of the Human Intestinal Microbial Ecosystem" (SHIME®) system have also shown significant dysbiosis of the microbial community following low-dose chlorpyrifos exposure (Joly et al., 2013). Stanaway et al. (2017) showed a significant association between the taxonomic composition of the human oral buccal microbiome with blood concentrations of the agricultural pesticide azinphos-methyl. However, as yet no studies have specifically investigated the association between the human gut microbiome and pesticide exposure. Human experimental studies of this topic are ethically dubious, and most pesticide exposure studies have focussed on overt health problems rather than exploring the nuanced gut microbial changes. However, as highlighted above, microbial changes are likely to have overarching health effects and should attract further attention. Although animal models are commonly applied to investigate the toxicity of pesticides on the gut microbiome, extrapolation of these findings to humans is questionable due to the lack of correlation often observed between the results of animal and human studies (Loan et al., 2015).

The majority of human pesticide exposure occurs via contaminated food and water sources (Damalas & Eleftherohorinos, 2011), however quantification of exposure via these routes is a great challenge, and as a result, exposure assessments can be highly inaccurate (Arcella et al., 2021). We know that agricultural workers using pesticides are likely to have a greater exposure to pesticides than the general population due to their enhanced inhalation, ingestion and dermal absorption as a result of direct contact with the substances (Hoppin et al., 2006). Previous exposure studies have demonstrated higher pesticide levels in farmers compared to non-farmers (Gooijer et al., 2019). Another study similarly showed a peak in glyphosate concentrations in the urine of agricultural workers following a spray event on the farm (Mesnage et al., 2002), indicating that occupational exposure is a likely determinant of pesticide concentrations within the body. Due to this enhanced exposure, we will recruit occupationally exposed farmers and individuals who are not occupationally exposed in order to maximise the exposure contrast within our study. We will characterise pesticide exposure by quantifying concentrations in urine, wristbands and hair from our study participants. These three approaches have all been validated as proxies of short- and medium-term pesticide exposure.

We have chosen to characterise the gut microbiome two days after acute exposure following a pesticide spray event. Although time series experiments have not been conducted in order to track the gut microbial changes in relation to pesticide exposure, data from previous studies have shown significant alterations in gut microbial species two days after antibiotic exposure in swine (K. Gao et al., 2018). In addition, a study of the human gut microbiome has shown that short-term dietary changes can trigger next-day shifts in gut microbial abundances (David et al., 2014). These results indicate that gut microbial alterations can occur rapidly after an acute exposure event and we aim to assess whether acute pesticide exposure has an impact on the gut microbial structure within two days. Our study is designed as a longitudinal cohort study of occupationally exposed and unexposed individuals with the primary aim to assess whether acute pesticide exposure has an impact on the structure of the human gut microbiome.

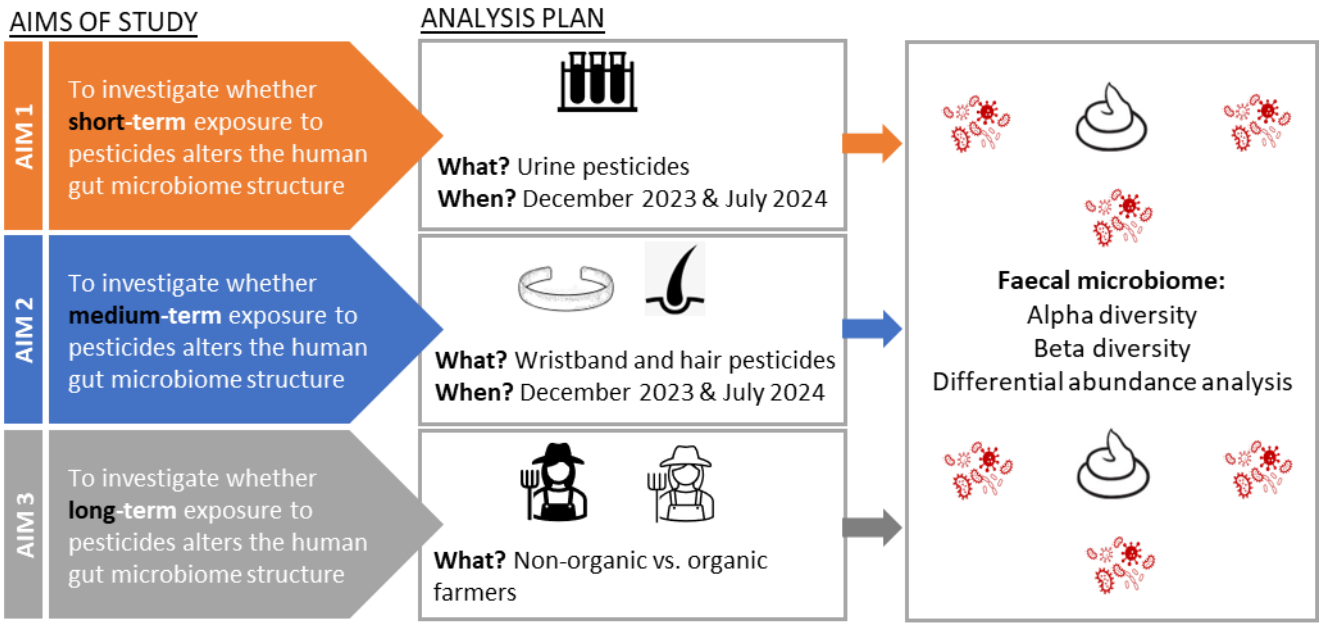
4. Study aims

The primary aims of the GuMPEx study are to determine whether short- and medium-term exposures to pesticides alter the structure of the human gut microbiome. This will be achieved by investigating the impacts of pesticide concentrations within urine, wristband and hair on the structure of the gut microbiome. In addition, we aim to determine whether long-term occupational exposure is associated with

alterations in the gut microbial structure by using the non-organic farmers as a proxy for long-term exposure. The main study aims and their analysis plans can be found in Figure 1.

Additional study objectives include an exploration of possible predictors of pesticide exposure. Questionnaire responses will be examined as potential predictors of urine, wristband and hair pesticide concentrations. In addition, due to the repeated gut microbial characterisations, we will be able to examine whether there are seasonal fluctuations of the gut microbiome.

Figure 1: Visual representation of the aims of the GuMPEx study and their associated analysis plans.



5. Methods

5.1 Study population

The proposed recruitment procedure of this study focusses on flower bulb farmers in the Netherlands as flower bulb cultivation has the highest pesticide intensity use in the country (Statistics Netherlands, 2022) and therefore these workers are likely to have particularly elevated occupational exposures. We will select both non-organic and organic flower bulb farmers in order to maximise exposure contrast within our study population. We know that there are several lifestyle factors associated with the farming occupation that are likely drivers of the gut microbiome, therefore comparing between groups of farmers enables us to control for some of these inherent factors. However, in order to generalise our results to the non-farming population, we will also include a group of residents in the neighbouring areas. Non-organic flower bulb farmers will be identified and contacted from databases of grown crops and land ownership. Organic flower bulb farmers will be identified and approached via associations of organic farmers such as the 'Natural Living and Growth' (NLG) Holland association (https://nlgholland.com/).

5.2 Exposure assessment

Pesticide exposure assessment within this cohort will be performed using several validated approaches as proxies for short-, medium- and long-term exposures. Exposure assessments will be conducted at two time points throughout the study: December 2023 and July 2024. The pesticide spray season is from March to September, therefore assessing exposure in July will likely capture high pesticide exposure within the non-

organic farmers. Assessing exposure at two time points will also enable us to determine whether seasonal fluctuations in exposure occur. A previous study demonstrated that pesticide spray events on non-organic farms are associated with increases in concentrations of measured pesticides within urine, wristbands and hair (Gooijer et al., 2019), therefore we expect to observe differences in the exposure estimates in December compared to July.

5.2.1 Short-term pesticide exposure

Concentrations of urine pesticide metabolites for five selected pesticides will be quantified using the five techniques as outlined in Gooijer et al. (2019) and used as a proxy for short-term exposure. Human experimental studies have shown that oral and dermal exposure to these five selected pesticides results in the excretion of five corresponding metabolites which will be selected for detection in urine samples (Gooijer et al., 2019). The five selected pesticides are: tebuconazole, chlorpropham, carbendazim, prochloraz and asulam and their associated metabolites are: tebuconazole-1-hydroxy (TEB-OH), 4-hydroxychlorpropham-O-sulphonic acid (4-HSA), methyl 5-hydroxy-2-benzimidazole carbamate (5-HBC, hydroxy-carbendazim), 2,4,6-trichlorophenoxyacetic acid (2,4,6-TCP) and asulam. Selection of these five pesticides within the Onderzoek Bestrijdingsmiddelen en Omwonenden (OBO) study was driven by several factors including their widespread usage in the study region in addition to their differing physicochemical properties and lower likelihood of dietary exposure compared to other pesticides (reducing the potential for confounding by dietary intake). We have reason to believe that exposure to each of these five pesticides is likely to cause gut microbial alterations. In relation to tebuconazole exposure, studies have shown significant impacts on soil microbial activity (Kent & Triplett, 2002). Chlorpropham is a carbamate ester similar in structure to the carbamate insecticide aldicarb which was shown by Gao et al. (2019) to cause significant disruptions in the gut microbiome of exposed mice. Carbendazim and prochloraz exposures in mice have also both been shown to induce dysbiosis of the gut microbiota (Jin et al., 2018; Y. Wang et al., 2021). Regarding asulam exposure, we know that its degradation product is sulfanilamide which is a sulphonamide class antibiotic (Kaufmann & Kaenzig, 2007), and is as such very likely to disrupt the gut microbial communities.

Within the OBO study, it was found that biomarkers of two out of these five selected for analysis were detected in more than half of all participant urine samples. Participants in this study included neighbouring residents to farms, in addition to a control population who lived at least 500m from an agricultural field. We therefore have confidence that we will detect these pesticides within our study population as our population will be more highly exposed than those involved in the OBO study. In order to determine overall urine pesticide concentration for each participant we will take an average (mean) of the five pesticides and express this in ng per mL of urine.

5.2.2 Medium-term pesticide exposure

Although urine sampling is an effective, convenient and non-invasive method to assess personal exposure to pesticides, it can only provide information on short-term pesticide exposure, with a maximum of several days (Tsatsakis et al., 2010). Urine is therefore a suitable matrix to assess exposure following specific spray events. However, it is likely that non-organic farmers have a different exposure profile to organic farmers, and are likely to experience longer-term pesticide exposures which may not be detected through urine analysis.

Although the highest likely exposures in an occupational setting are episodic following spray events, it is known that farmers are exposed to long-term, low-level exposures through other activities that may not be directly associated with the spray event. These exposures include contact with pesticide residues on the

crops and take-home exposures (Fenske et al., 2013), in addition to spray drift from neighbouring fields (Damalas & Koutroubas, 2016). These additional exposures highlight the fact that we should not solely rely on short-term estimates of pesticide exposure. Furthermore, there are limitations on the number and type of pesticides that we are able to analyse in the urine samples due to a lack of analytical standards for biomarkers of many of the pesticides used within this sector, hence limiting our urine biomarker analysis to only five pesticide biomarkers. Therefore, in addition to urine analysis we also plan to create an enhanced understanding of medium-term pesticide exposure to a wider selection of pesticides by analysis of silicone wristbands (worn by the farmers) and hair samples.

All farmers will be asked to wear a silicone wristband continuously for 1 week both in the non-spraying and spraying seasons (December 2023 and July 2024 respectively). Wristbands are a practical method to assess medium-term exposure to pesticides as it is reasonable to implement and non-invasive for the participants involved (Gooijer et al., 2019). These wristbands adsorb chemicals such as pesticides in their matrix which can then be analysed. Wristbands will be analysed for 46 different targeted pesticides or their breakdown products. These 46 pesticides were previously selected as targets in the OBO study as they are known to be commonly used in flower bulb production (Gooijer et al., 2019). Pesticides will be measured using a multi-residue method which will allow for all selected pesticides to be simultaneously quantified. This method will be based on liquid chromatography linked with mass spectrometry (LC-MS/MS) (see Gooijer et al. (2019) for further details). Pesticide concentrations in the wristbands will be expressed in ng per g of wristband. Hair samples will also be collected from all farmers (where possible) both in the spray and non-spray seasons, following the protocol defined by Cooper et al. (2012). Hair has been found to be a suitable matrix for biomonitoring of human environmental exposure to pesticides (Appenzeller & Tsatsakis, 2012). Hair samples will be analysed using the LC-MS/MS-based method. However, due to the fact that hair is a complex matrix which can be highly variable, isotopically labelled internal standard analogues for the pesticides of interest will be required to reduce the analytical errors when determining pesticide quantities (Soulard et al., 2022; Tsuchiyama et al., 2017). As a result, we will not be able to target the same 46 pesticides as those in the wristbands but instead we will target 25 specific pesticides. These 25 specific pesticides were selected firstly to match with the five pesticides targeted in the urine analyses and the remaining 20 pesticides were selected due to their high prevalence in the environmental samples collected as part of the OBO study which was conducted in a similar region to this proposed study (Gooijer et al., 2019). For estimations of overall pesticide levels in each matrix, we will take the mean of the concentrations for each of the quantified pesticides and use these concentrations as continuous variables in our analyses.

5.2.3 Long-term pesticide exposure

Over their lifetimes, we know that farmers who use pesticides are exposed to much higher levels than the general public (Woodruff et al., 1994). Therefore we will broadly classify non-organic farmers as individuals experiencing long-term pesticide exposure.

A summary of the short-, medium- and long-term pesticide exposure assessment methods that will be implemented can be found in Table 1.

Table 1: Methods used to determine short- and long-term pesticide exposure

Sample/data type	Collection time (length)	Analysis method
Urine	December 2023 (1 morning sample) July 2024 (1 morning sample)	5 different detection methods for the 5 pesticide targets (see Gooijer et al. (2019))
Wristband	December 2023 (7 days) July 2024 (7 days)	LC-MS/MS (46 pesticide targets)
Hair	December 2023 (1 sample) July 2024 (1 sample)	LC-MS/MS (25 pesticide targets)

5.2.4 Participant questionnaires

In order to explore possible lifestyle-related determinants of the above measured pesticide concentrations, questionnaires will be conducted and will consist of a set of questions related to both direct and indirect pesticide exposures. Participants will complete questionnaires directly in the study database. Non-organic farmers will be asked questions regarding pesticide use on their fields, including information on the types of pesticides, spraying methods, quantities and times of spraying as well as information on personal protective equipment used. All participants will be asked about food consumption, personal use of pesticides at the home, lifestyle information and actions that may be involved with enhanced pesticide exposure. Questionnaires will be completed twice during the study to coincide with the exposure measurements and microbiome sampling.

5.3 Microbiome (outcome) processing

Faecal samples will be collected by the participants in December 2023 and July 2024. Participants will be asked to store samples in the fridge until collection by the coordinating team the following day. On return to the lab, samples will be stored in the -80°C freezer prior to sequencing. We will perform 16S rRNA gene amplicon sequencing of faecal samples from all participants. DNA will firstly be extracted from faecal samples and then amplified using specific primers targeting the V3 and V4 regions of the bacterial 16S rRNA gene. Subsequently, sequencing will be conducted using the Illumina MiSeq platform. Amplicon data analysis will be performed using the QIIME pipeline (Bolyen et al., 2019). This involves filtering out artifacts such as primers and barcodes as well as low-quality reads, and subsequently determining the counts of non-redundant sequences (Qian et al., 2020). Output sequences will be processed in R and the sequences will be grouped into amplicon sequence variants (ASVs) (Callahan et al., 2016). For taxonomic classification, sequences will be mapped to the SILVA database which is a comprehensive dataset containing aligned rRNA gene sequences (Quast et al., 2013). Additional filtering and quality checks of the microbial dataset will be performed. Firstly contaminant DNA will be identified using the *decontam* package in R and sequences deemed to be contaminants with the 'combined' method will be removed. In addition, bacterial taxa with an abundance of <0.1% in <1% of all samples will be removed.

5.4 Statistical analyses

5.4.1 Exposure characterisation

Descriptive statistics of the pesticides detected in urine, wristbands and hair will be reported. Tables will show a summary of the findings including the minimum, median, mean, and maximum values for each of the pesticide targets across all samples from the same matrix. We will also determine the percentage of samples from each matrix with values greater than the limit of detection (i.e. the lowest pesticide concentration that can be detected in a sample) for each pesticide.

5.4.1.1 Determinants of pesticide exposure

We will conduct regression analysis to investigate whether variations in urine, wristband and hair pesticide concentrations can be explained by predictors obtained from questionnaire results. This will enable us to determine possible lifestyle-related predictors of human pesticide exposure.

5.4.2 Microbiome characterisation

Several characteristics of the faecal microbiome will be explored within this study. All analyses will be performed in R. A more detailed statistical analysis plan (SAP) will be developed and published prior to initiation of the proposed study in order to protect from *p* hacking from post-hoc selection of diversity indices. A brief description of the analysis methods to be used is provided here.

5.4.2.1 Alpha diversity

We will explore within-sample diversity (alpha diversity) of each microbiome sample. We will use the R package *microbiome* to calculate three different alpha diversity indices (Chao1, Shannon and Simpson) for each sample, as these are commonly used in conjunction within human microbiome analyses. Each of these indices quantifies alpha diversity with a slightly different emphasis (Chen & Chen, 2018). Alpha diversity can be classified according to richness (the number of different taxa) or evenness (the distribution of taxa abundances). Chao1 quantifies diversity based only on richness, Shannon and Simpson indices both combine richness and evenness but Shannon gives more weight to rare species whereas Simpson gives more weight to common species (Qian et al., 2020).

Chao1, Shannon and Simpson alpha diversity indices will be our outcome variables. Primary explanatory variables of interest will be pesticide exposure concentrations from urine, wristbands and hair samples. In addition, other participant characteristic variables such as age, sex, season (December vs. July), diet (low, medium and high likelihoods of pesticide consumption), personal use of pesticides (yes vs. no) will be explored as possible determinants of the microbiome. Statistical differences in alpha diversity will be assessed using multivariable linear models for the continuous concentration exposure variables. For categorical participant characteristic variables, we will initially explore these factors univariably (using *t* tests, Wilcoxon rank sum tests and ANOVA). Following this, predictors yielding *p* values <0.2 will be entered into the multivariable linear models with the pesticide exposure concentrations to allow for adjustment for these known confounders.

5.4.2.2 Beta diversity

Beta diversity quantifies compositional differences between samples and allows us to determine and visualise whether or not microbiome compositions vary between and within individuals that are grouped according to a variable of interest. Bray-Curtis (BC) dissimilarity will be calculated between all microbiome samples using the *vegan* R package (Oksanen et al., 2007). Principal Coordinates Analysis (PCoA) will subsequently be used to visualise these dissimilarities in a two-dimensional plot. We are primarily interested to see whether urine pesticide concentrations are associated with compositional changes in the gut microbiome, we firstly categorise all participants into quartiles of exposure based on total urine pesticide concentrations. In addition, we again categorise participants into quartiles for the medium-term pesticide exposures (wristband and hair concentrations). In order to statistically test whether there are overall differences between microbial compositions of participants in different quartiles of pesticide exposure (in urine, wristbands and hair), we will use a multivariable permutational multivariate analysis of variance (PERMANOVA) (*adonis2* function), along with a check for homogeneity of variance between the quartiles (*betadisper* function).

5.4.2.3 Differential abundance analysis

Differential abundance (DA) methods are able to detect associations between bacterial abundances and participant groupings (Hawinkel et al., 2019). Within this study, we will use this analysis to determine whether there are specific bacterial taxa that are driving potential compositional differences between individuals exposed to different levels of pesticides, hence determining whether there are specific bacterial taxa that have significantly higher or lower abundances with increases in pesticide exposure. We will implement two different DA analysis algorithms in order to validate our results as different methods have been shown to produce inconsistent results (Nearing et al., 2022). We will implement the DESeq and ALDEx2 algorithms as previous studies have recommended that they are used in conjunction for DA analysis of microbial communities (Nearing et al., 2022).

5.4.2.4 Procrustes analysis

In order to investigate whether the gut microbiome is stable over time within one individual we will use Procrustes analysis. This allows us to compare the correlations between the PCoA ordinations of the gut microbiome samples from December 2023 to those from July 2024 within the same individual. In order to determine whether or not potentially observed correlations between paired samples are true correlations and not due to chance, we will also randomise the December 2023 samples and compare these to their unrelated July 2024 samples. This will enable us to determine the within and between-person microbial dissimilarities over time, hence enabling us to decipher the stability of the gut microbiome over time. Procrustes analysis will be implemented using the R *vegan* package. Firstly, PCoA ordinations of all gut microbiome samples will be computed using the BC dissimilarity matrix. Subsequently, PCoA ordinations of the paired and unpaired microbiomes will be superimposed in a symmetric Procrustes plot. The *protest* function from *vegan* will be used to statistically test the significance of the Procrustes correlation between ordinations and a p value of <0.05 will be considered statistically significant.

5.5 Study timelines

The GuMPEx study will be conducted between August 2022 and March 2025 in multiple stages. The activities and schedule status of the GuMPEx study is illustrated in a Gantt chart in Figure 2, and a detailed overview of the planned study activities within these 32 months can be seen in Figure 3.

Figure 2: Gantt chart to illustrate the GuMPEx study schedule.

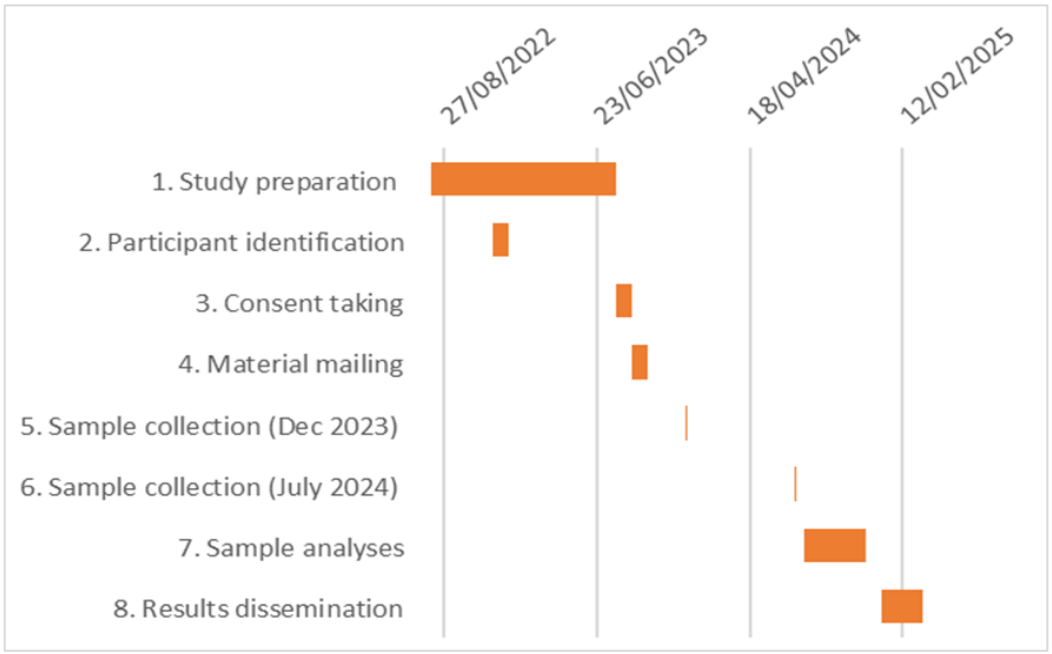
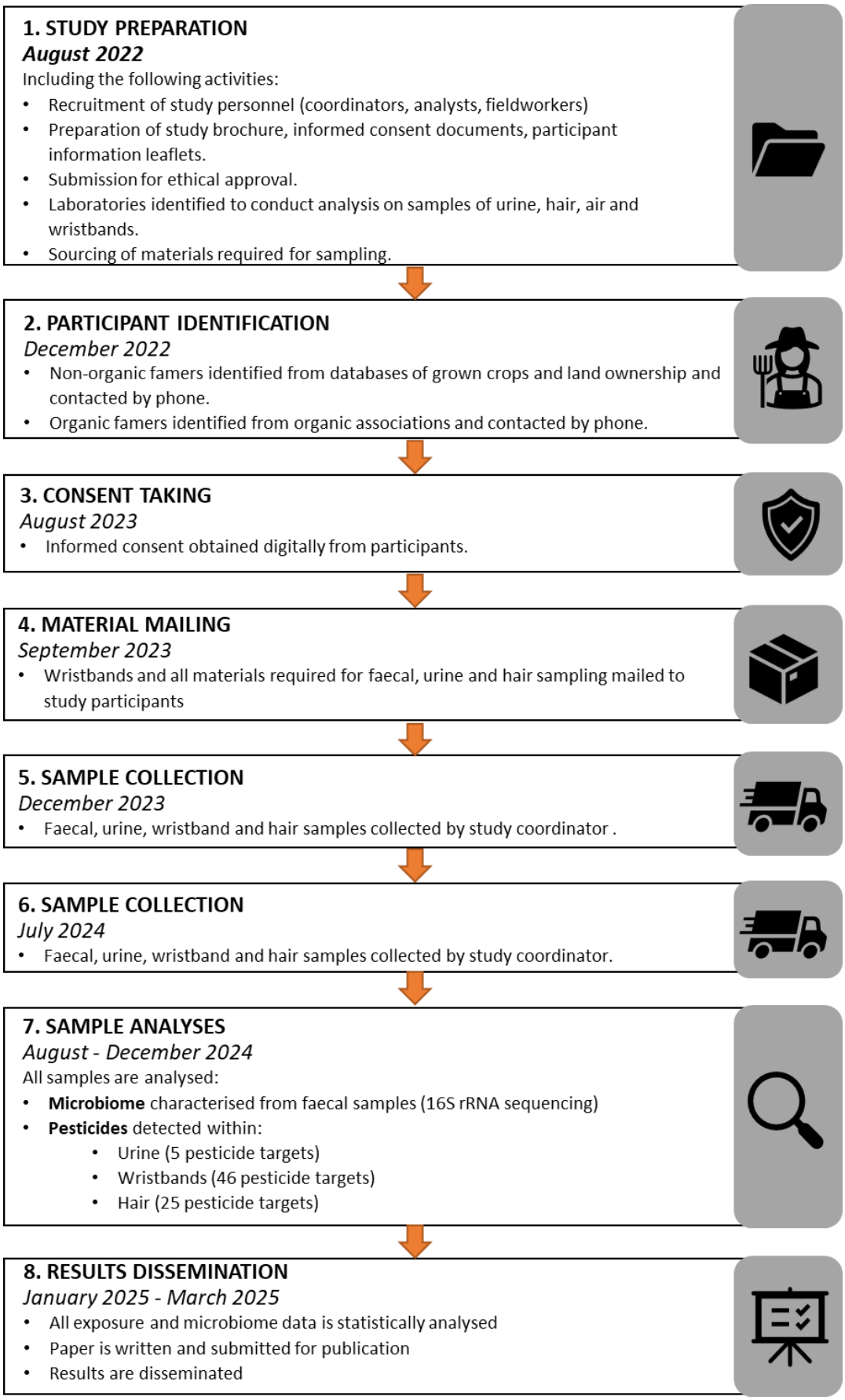


Figure 3: Overview of the study tasks and timelines.



5.6 Power calculation

There are some power calculation tools which are specific to microbiome analyses, however regular sample size procedures are also applicable for microbiome data. The G*Power software (version 3.1.9.7) will be used to determine the appropriate sample size required to meet our study aims (Faul et al., 2007), which is known to be the most complete software for power analysis. The microbiome is a complex structure and two main aspects of it can be compared between individuals: alpha and beta diversity (the within and between-sample diversity respectively). Kers & Saccenti (2022) have shown that beta diversity metrics were more sensitive to detection of differences in comparison to alpha diversity metrics. In addition, they observed that the different types of alpha and beta diversity metric also have an impact on the sample size required to detect differences. In terms of beta diversity metrics, they showed that the BC index is the most sensitive. They found that the microbiome structure had an influence on the sensitivity of the different alpha diversity indices, and that with human microbiome data the Shannon and Simpson alpha diversity indices were more sensitive than Observed, Chao1 and Phylogenetic Diversity indices (Kers & Saccenti, 2022). As we plan to characterise alpha diversity using Chao1, Shannon and Simpson indices, we will conduct our power calculation using Chao1 as our outcome measure, as this is the least sensitive of the analyses we will perform on our microbiome data. We will be determining whether there is a linear relationship between pesticide exposure concentrations and the Chao1 alpha diversity index, therefore our sample size calculation will be based on a *t* test to assess the linearity of the relationship between Chao1 diversity and urine pesticide concentrations by assessing slope size. In order to compute the required sample size, the following parameters must be specified in the G*Power software: α (type I error rate), power (1 – β (type II error rate)), population standard deviation and effect size.

Within the G*Power algorithm, we will specify $\alpha = 0.05$ and power = 0.8. However the difficulty lies in determining the hypothesised effect size of pesticide exposure on Chao1, in addition to the challenge of estimating the variability of Chao1 scores within our study population. We expect that Chao1 diversity will decrease linearly with increases in pesticide concentration. However, due to the varying intensity of effects observed as a result of different environmental exposures on microbial diversity (Alderete et al., 2018; Lei et al., 2019), estimating effect size would be mere speculation and could drastically influence the outcome of our power analysis. Therefore, simply estimating effect size and variability could lead to a gross overestimation or underestimation of the required sample size for this study. As a result, we will conduct a small pilot microbiome analysis to help us to determine reasonable estimations of these figures. We will approach a selection of previous participants from the OBO study (Gooijer et al., 2019) for faecal samples. Pesticide exposures for these participants were previously determined, therefore we will not only be able to make informed estimations of Chao1 variability within the population, but also informed estimations of the effect size in relation to pesticide exposure.

As we will conduct linear regression to determine whether there is a significant linear relationship present here, therefore we express this relationship with Equation 1. As follows, our null hypothesis (H_0) and (two-sided) alternative (H_1) hypothesis are presented in Equation 2 (Seltman, 2018).

Equation 1: $E(Y|x) = \beta_0 + \beta_1 x$

Equation 2: $H_0: \beta_1 = 0$, $H_1: \beta_1 \neq 0$

Where Y is the alpha diversity index (e.g. Chao1), x is the (continuous) pesticide concentration, $E(Y|x)$ is the expected value of the diversity index given the pesticide concentration. β_0 and β_1 are the intercept

For financial and staff budgeting purposes, we assume a total cohort of 60 within this study which will include non-organic and organic farmers as well as a group of non-farming individuals. This corresponds with sample sizes of similar studies previously conducted, such as that of Yang et al. (2019) who revealed significant alterations in gut microbial diversity following phthalate exposure with a cohort of 75.

6. Expected results and societal implications

Despite the growing body of evidence showing that gut microbial alterations are associated with many disease states, moving from correlation to causation is a challenge within microbiome research (Shreiner et al., 2015). Interindividual variability of the gut microbial communities additionally convolutes the picture and adds a further challenge in the determination of the microbial drivers of health and disease. However, we have considerable evidence now to show that reduced gut microbial diversity is linked with several chronic health conditions. Therefore, by revealing the impacts of pesticide exposure on microbial diversity, we can speculate whether or not pesticides may be modulating health status. Currently, in terms of policies regarding pesticide approval, review processes do not investigate alterations in the gut microbiome but instead focus on overt health conditions. Despite the fact that gut microbial alterations cannot be explicitly linked with health conditions, we believe that it should be considered within the review process as they can provide early warning signs for progression of several chronic diseases. We hope to reveal initial evidence to show that gut microbial changes are associated with pesticide exposures, which we hope will provide stimulus for further investigations within this field. In terms of policy making, we hope that our study will provide a stimulus to consider this as an outcome of interest in relation to risk assessment of these substances.

7. Risk assessment and ethical considerations

Due to the observational nature of this study, we do not anticipate significant hazards that could be of harm to our study participants. There are however several potential risks, biases and ethical considerations that must be taken into account through the design and conduct of this study. Ethical approval for the study will be sought from the medisch ethische toetsingscommissie (METC) of the Utrecht Medical Centre at Utrecht University.

7.1 Risk of low participation rates

Despite the minimal physical risks to participants, our study is demanding of time and energy from those involved. All participants will be required to complete questionnaires, wear wristbands and provide urine, faecal and hair samples for the study analysis. For many approached farmers, this may appear excessively demanding, and as a result we foresee risks of low participation and adherence rates. To attempt to overcome this, we will provide financial incentives for participation in the study in addition to regular communication with participants and provision of study giveaways. We trust that incentivisation to partake in our study will not introduce ethical concerns related to coercion and exploitation of vulnerable groups due to the lack of physical hazards from our study.

7.2 Risk of selection and confounding bias

There is a possibility of the introduction of selection bias within our study via self-selection into the study. It could be that the non-organic farmers who agree to participate in the study have prior concerns regarding their health and the health effects of pesticide use on the farm. They therefore may be more likely to have worse health status which could possibly be linked to alterations in the gut microbiome, which may or may not be linked to pesticide exposure. Although little can be done to prevent selection bias from initially occurring, we will minimise the impacts of this possible bias by the use of extensive questionnaires which will investigate many lifestyle and health factors. We will correct for characteristics such as age, sex and health status which are all known to be key drivers of gut microbial structure.

7.3 Risk of information bias

Systematic errors in measurements of the outcome (gut microbiome) are not expected within our study. These will be mitigated by pseudonymising participants which will ensure that lab analyses are conducted blind to the exposure status of the farmer under investigation. All gut microbiome analyses will be

conducted using standardised 16S rRNA sequencing protocols and all analyses will be performed in the same laboratory for consistency. In addition, all samples will be 16S rRNA sequenced within the same sequencing run in order to reduce batch effects which often occur as a result of conducting independent sequencing runs due to possible variations in hardware, reagents, or personnel (Schloss et al., 2011). We do not foresee any introduction of bias regarding exposure assessment in our study. All samples from farmers will be collected following standardised data collection protocols, and participants will be asked to fill out questionnaires as truthfully as possible and reminded that their responses will be pseudonymised.

7.4 Informed consent process

All study participants will be approached for fully informed consent in August 2023. Participants will be provided with personal access to the study database from which they will read the information sheet and consent form as developed for the study and approved by the METC. Before accessing the consent form, participants will be required to read the information sheet (see Appendix 10.1). This will contain information such as details of the study, risks and benefits of participation, details about withdrawal from the study and information regarding the future use and storage of the study data. Consent forms will consist of checkboxes enabling the participant to indicate agreement to the conditions of the study (see Appendix 10.2). The consent form will include a statement asking whether the participant agrees in the notification of their general practitioner and the Gemeentelijke Gezondheidsdienst (GGD) in the case that their pesticide exposure exceeds the acceptable daily intake. A copy of the information leaflet and completed consent form will be provided to the consenting participant for their records and the original copy will be stored electronically in the study database. No copies of the consent form will be stored elsewhere in order to protect participants' identities.

7.5 Participant confidentiality

In order to protect the privacy of our study participants and minimise the risk of data breaches, all information and sample analyses from participants will be stored securely in an encrypted study database. Questionnaire responses will be directly entered onto the study database by the participants themselves. Pseudonymisation of personal data will be carried out in order to protect the privacy of participants, but to enable withdrawal of participant data if necessary.

7.6 Results dissemination

We aim to publish the results arising from the GuMPEx study in a well-established, peer-reviewed journal. In addition to publication, study results will be provided to participants and other stakeholders involved via dissemination packs which will include several materials including participant leaflets, a copy of the publication, and their personal exposure measurements when requested. We will approach various media outlets such as the bulb growers' magazines 'Greenity', and other national farming-related journals. We will arrange a participant and stakeholder meeting in March 2025 where the results of the study and their implications will be discussed in further detail.

8. Project budgeting

We plan to initiate this study in August 2022 and we expect to complete and disseminate the results by March 2025. We will employ several staff members with varying roles in the study. The principal investigator will be responsible for overall management of the study and staff, and will be involved in the design, conduct and reporting of the study results as well as being a point of contact for collaborative partnerships with stakeholders within the project. The research associate will be responsible for the coordination of the team, have overall responsibility for the project timelines and will be in charge of monitoring of the data collection process. In addition, the research associate will design the statistical analysis plan, conduct statistical analyses and will have overall responsibility for the writing of the publication. The data manager will develop the study database and will have overall responsibility of it,

ensuring accuracy and legitimacy of the data as well as ensuring that all practices are compliant with the European Union's General Data Protection Regulation (EU GDPR). Fieldworkers will be responsible for all communication with the study participants, for mailing study materials to, and collecting samples from the study participants. Lab technicians will be recruited to conduct the analyses of all samples collected. Four different labs will be involved in the study due to the different methods and materials required in order to process the different types of samples from participants. The communications officer will be employed from the university as an independent figure to advise and implement various communication strategies. The financial budget plan of the study is outlined in Table 2.

Table 2: Financial plan of the GuMPEx study.

Job title	NFU/VSNU member	Salary Scale*	Months	Gross salary	% FTE[±]	Salary costs
Principal investigator	VSNU	Senior Scientific Employee	32	€221,262	20%	€44,252.40
Research associate	VSNU	Senior Scientific Employee	32	€221,262	100%	€221,262.00
Data manager	VSNU	NSE[‖] - Academic	32	€236,985	60%	€142,191.00
Fieldworker	VSNU	NSE - MBO[¥]	3	€13,976	100%	€13,976.00
Fieldworker	VSNU	NSE - MBO	3	€13,976	100%	€13,976.00
Lab Technician	VSNU	NSE - MBO	5	€23,293	80%	€18,634.40
Lab Technician	VSNU	NSE - MBO	5	€23,293	80%	€18,634.40
Lab Technician	VSNU	NSE - MBO	5	€23,293	80%	€18,634.40
Lab Technician	VSNU	NSE - MBO	5	€23,293	80%	€18,634.40
Communications officer	VSNU	NSE - MBO	2	€9,317	50%	€4,658.50
TOTAL STAFF COSTS						**€514,853.50**

Item Category	Item	Cost per item	Number	Total
Sample analyses	Faecal 16S rRNA sequencing	€65	120	€7,800
	Urine pesticide analysis	€150	120	€18,000
	Wristband analysis	€250	120	€30,000
	Hair sample analysis	€250	120	€30,000
Materials	Materials for urine, faecal and hair sampling	€10	120	€1,200
	Wristbands	€10	120	€1,200
	Fieldworker travel costs			€500
	Printing of study materials			€600
Participants	Phone bills			€250
	Financial incentives	€200	60	€12,000
Other	Database development			€6,000
	Secure sample storage			€1,000
TOTAL OTHER STUDY COSTS				**€108,550**
GRAND TOTAL				**€623,403.50**

* Salaries have been determined based on the salary tables agreed upon by the collective labour agreement of the Dutch universities (Universities of the Netherlands, 2022). The salary budgeting table has been adapted from The Netherlands Organisation for Health Research and Development (ZonMw) template (https://www.zonmw.nl/nl/subsidies/voorwaarden-en-financien/).

[±] % FTE is the % of full-time equivalent work (defined as 38 hours per week) that an employee will work on the project.

[‖] NSE is the acronym for Non Scientifical Employee.

[¥] MBO is the Dutch acronym for senior secondary vocational education.

9. References

Alderete, T. L., Jones, R. B., Chen, Z., Kim, J. S., Habre, R., Lurmann, F., Gilliland, F. D., & Goran, M. I. (2018). Exposure to traffic-related air pollution and the composition of the gut microbiota in overweight and obese adolescents. *Environmental Research*, *161*(September 2017), 472–478. https://doi.org/10.1016/j.envres.2017.11.046

Appenzeller, B. M. R., & Tsatsakis, A. M. (2012). Hair analysis for biomonitoring of environmental and occupational exposure to organic pollutants: State of the art, critical review and future needs. *Toxicology Letters*, *210*(2), 119–140. https://doi.org/10.1016/j.toxlet.2011.10.021

Arcella, D., Cascio, C., Dujardin, B., Gergelová, P., Ángel, J., Ruiz, G., Horváth, Z., Ioannidou, S., Pastor, P. M., & Riolo, F. (2021). *Technical report on handling occurrence data for dietary exposure assessments*. *December*. https://doi.org/10.2903/sp.efsa.2021.EN-7082

Asghar, U., Malik, M., & Javed, A. (2016). Pesticide Exposure and Human Health: A Review. *Journal of Ecosystem & Ecography*, *01*(s5). https://doi.org/10.4172/2157-7625.s5-005

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Chen, J., & Chen, D.-G. (2018). *Statistical Analysis of Microbiome Data with R - ICSA Book Series in Statistics*. http://www.springer.com/series/13402

Cooper, G. A. A., Kronstrand, R., & Kintz, P. (2012). Society of Hair Testing guidelines for drug testing in hair. *Forensic Science International*, *218*(1–3), 20–24. https://doi.org/10.1016/j.forsciint.2011.10.024

Damalas, C. A., & Eleftherohorinos, I. G. (2011). *Pesticide Exposure , Safety Issues , and Risk Assessment Indicators*. 1402–1419. https://doi.org/10.3390/ijerph8051402

Damalas, C. A., & Koutroubas, S. D. (2016). Farmers' exposure to pesticides: Toxicity types and ways of prevention. *Toxics*, *4*(1), 1–10. https://doi.org/10.3390/toxics4010001

David, L. A., Materna, A. C., Friedman, J., Campos-Baptista, M. I., Blackburn, M. C., Perrotta, A., Erdman, S. E., & Alm, E. J. (2014). Host lifestyle affects human microbiota on daily timescales. *Genome Biology*, *15*(7), 1–15. https://doi.org/10.1186/gb-2014-15-7-r89

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*, *39*(2), 175–191. https://doi.org/10.3758/bf03193146

Fenske, R. A., Lu, C., Negrete, M., & Galvin, K. (2013). Breaking the Take Home Pesticide Exposure Pathway for Agricultural Families: Workplace Predictors of Residential Contamination. *Am J Ind Med.*, *56*(9), 1063–1071. https://doi.org/10.1002/ajim.22225

Fetissov, S. O. (2017). Role of the gut microbiota in host appetite control: Bacterial growth to animal feeding behaviour. *Nature Reviews Endocrinology*, *13*(1), 11–25.

https://doi.org/10.1038/nrendo.2016.150

Fouladi, F., Bailey, M. J., Patterson, W. B., Sioda, M., Blakley, I. C., Fodor, A. A., Jones, R. B., Chen, Z., Kim, J. S., Lurmann, F., Martino, C., Knight, R., Gilliland, F. D., & Alderete, T. L. (2020). Air pollution exposure is associated with the gut microbiome as revealed by shotgun metagenomic sequencing. *Environment International*, *138*(March), 105604. https://doi.org/10.1016/j.envint.2020.105604

Gao, B., Chi, L., Tu, P., Gao, N., & Lu, K. (2019). The Carbamate Aldicarb Altered the Gut Microbiome, Metabolome, and Lipidome of C57BL/6J Mice. *Chemical Research in Toxicology*, *32*(1), 67–79. https://doi.org/10.1021/acs.chemrestox.8b00179

Gao, K., Pi, Y., Peng, Y., Mu, C. L., & Zhu, W. Y. (2018). Time-course responses of ileal and fecal microbiota and metabolite profiles to antibiotics in cannulated pigs. *Applied Microbiology and Biotechnology*, *102*(5), 2289–2299. https://doi.org/10.1007/s00253-018-8774-2

Gooijer, Y. M., Hoftijser, G. W., Lageschaar, L. C. C., Oerlemans, A., Scheepers, P. T. J., Kivits, C. M., Duyzer, J., Gerritsen-Ebben, M. G., Figueiredo, D. M., Huss, A., Krop, E. J. M., Vermeulen, R. C. H., van den Berg, F., Holterman, H. J., Jacobs, C. J. M., Kruijne, R., Mol, J. G. J., Wenneker, M., van de Zande, J. C., & Sauer, P. J. J. (2019). *Research on exposure of residents to pesticides in the Netherlands*. 318. https://edepot.wur.nl/475219

Hawinkel, S., Mattiello, F., Bijnens, L., & Thas, O. (2019). A broken promise: Microbiome differential abundance methods do not control the false discovery rate. *Briefings in Bioinformatics*, *20*(1), 210–221. https://doi.org/10.1093/bib/bbx104

Hoppin, J. A., Adgate, J. L., Eberhart, M., Nishioka, M., & Ryan, P. B. (2006). Environmental exposure assessment of pesticides in farmworker homes. *Environmental Health Perspectives*, *114*(6), 929–935. https://doi.org/10.1289/ehp.8530

Human Microbiome Project Consortium. (2014). The integrative human microbiome project: Dynamic analysis of microbiome-host omics profiles during periods of human health and disease corresponding author. *Cell Host and Microbe*, *16*(3), 276–289. https://doi.org/10.1016/j.chom.2014.08.014

Jin, C., Zeng, Z., Wang, C., Luo, T., Wang, S., Zhou, J., Ni, Y., Fu, Z., & Jin, Y. (2018). Insights into a possible mechanism underlying the connection of carbendazim-induced lipid metabolism disorder and gut microbiota dysbiosis in mice. *Toxicological Sciences*, *166*(2), 382–393. https://doi.org/10.1093/toxsci/kfy205

Joly, C., Gay-Quéheillard, J., Léké, A., Chardon, K., Delanaud, S., Bach, V., & Khorsi-Cauet, H. (2013). Impact of chronic exposure to low doses of chlorpyrifos on the intestinal microbiota in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) and in the rat. *Environmental Science and Pollution Research*, *20*(5), 2726–2734. https://doi.org/10.1007/s11356-012-1283-4

Karlsson, F. H., Tremaroli, V., Nookaew, I., Bergström, G., Behre, C. J., Fagerberg, B., Nielsen, J., & Bäckhed, F. (2013). Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature*, *498*(7452), 99–103. https://doi.org/10.1038/nature12198

Kaufmann, A., & Kaenzig, A. (2007). Contamination of honey by the herbicide asulam and its antibacterial active metabolite sulfanilamide. *Food Additives & Contaminants*, *21*(6), 564–571. https://doi.org/10.1080/02652030410001677790

Kent, A. D., & Triplett, E. W. (2002). Microbial communities and their interactions in soil and rhizosphere ecosystems. *Annu. Rev. Microbiol.*, *56*, 211–236.

Kers, J. G., & Saccenti, E. (2022). The Power of Microbiome Studies: Some Considerations on Which Alpha and Beta Metrics to Use and How to Report Results. *Frontiers in Microbiology*, *12*(March), 1–18. https://doi.org/10.3389/fmicb.2021.796025

Lei, M., Menon, R., Manteiga, S., Alden, N., Hunt, C., Alaniz, R. C., Lee, K., & Jayaraman, A. (2019). Environmental chemical diethylhexyl phthalate alters intestinal microbiota community structure and metabolite profile in mice. *AIChE Annual Meeting, Conference Proceedings*, *2019-Novem*(December), 1–16. https://doi.org/10.1128/msystems.00724-19

Lloyd-Price, J., Mahurkar, A., Rahnavard, G., Crabtree, J., Orvis, J., Hall, A. B., Brady, A., Creasy, H. H., McCracken, C., Giglio, M. G., McDonald, D., Franzosa, E. A., Knight, R., White, O., & Huttenhower, C. (2017). Strains, functions and dynamics in the expanded Human Microbiome Project. *Nature*, *550*(7674), 61–66. https://doi.org/10.1038/nature23889

Loan, T., Nguyen, A., Vieira-silva, S., Liston, A., & Raes, J. (2015). *How informative is the mouse for human gut microbiota research ?* 1–16. https://doi.org/10.1242/dmm.017400

Lu, K., Abo, R. P., Schlieper, K. A., Graffam, M. E., Levine, S., Wishnok, J. S., Swenberg, J. A., Tannenbaum, S. R., & Fox, J. G. (2014). Arsenic exposure perturbs the gut microbiome and its metabolic profile in mice: An integrated metagenomics and metabolomics analysis. *Environmental Health Perspectives*, *122*(3), 284–291. https://doi.org/10.1289/ehp.1307429

Mesnage, R., Moesch, C., Grand, R. Le, Lauthier, G., Vendômois, J. S. de, Gress, S., & Gilles-Eric, S. (2002). Glyphosate Exposure in a Farmer's Family. *American Journal of Human Genetics*, *70*(5), 1077–1088. https://doi.org/10.1086/340363

National Academies of Sciences Engineering and Medicine. (2018). Environmental Chemicals, the Human Microbiome, and Health Risk: A Research Strategy. In *Environmental Chemicals, the Human Microbiome, and Health Risk: A Research Strategy*. Washington, DC: The National Academies Press. https://doi.org/https://doi.org/10.17226/24960

Nearing, J. T., Douglas, G. M., Hayes, M. G., MacDonald, J., Desai, D. K., Allward, N., Jones, C. M. A., Wright, R. J., Dhanani, A. S., Comeau, A. M., & Langille, M. G. I. (2022). Microbiome differential abundance methods produce different results across 38 datasets. *Nature Communications*, *13*(1), 1–31. https://doi.org/10.1038/s41467-022-28034-z

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P. R., O'hara, R. B., Simpson, G. L., Solymos, P., Henry, M., Stevens, H., Szoecs, E., & Maintainer, H. W. (2007). *Package "vegan" Title Community Ecology Package*.

Qian, X. B., Chen, T., Xu, Y. P., Chen, L., Sun, F. X., Lu, M. P., & Liu, Y. X. (2020). A guide to human microbiome research: Study design, sample collection, and bioinformatics analysis. *Chinese Medical Journal*, *133*(15), 1844–1855. https://doi.org/10.1097/CM9.0000000000000871

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), 590–596. https://doi.org/10.1093/nar/gks1219

Ramirez, J., Guarner, F., Bustos Fernandez, L., Maruy, A., Sdepanian, V. L., & Cohen, H. (2020). Antibiotics as Major Disruptors of Gut Microbiota. *Frontiers in Cellular and Infection Microbiology*, *10*(November), 1–10. https://doi.org/10.3389/fcimb.2020.572912

Reygner, J., Condette, C. J., Bruneau, A., Delanaud, S., Rhazi, L., Depeint, F., Abdennebi-Najar, L., Bach, V., Mayeur, C., & Khorsi-Cauet, H. (2016). Changes in composition and function of human intestinal

microbiota exposed to chlorpyrifos in oil as assessed by the SHIME® model. *International Journal of Environmental Research and Public Health*, *13*(11), 1–18. https://doi.org/10.3390/ijerph13111088

Rodrigues, M. de B., Carvalho, D. S. de, Chong-Silva, D. C., Urrutia-Pereira, M., Albuquerque, G. S. C. de, Cieslak, F., & Chong-Neto, H. J. (2022). Association between exposure to pesticides and allergic diseases in children and adolescents: a systematic review with meta-analysis. *Jornal de Pediatria*, *000*(xxx). https://doi.org/10.1016/j.jped.2021.10.007

Schloss, P. D., Gevers, D., & Westcott, S. L. (2011). Reducing the effects of PCR amplification and sequencing Artifacts on 16s rRNA-based studies. *PLoS ONE*, *6*(12). https://doi.org/10.1371/journal.pone.0027310

Seltman, H. J. (2018). Chapter 9: Simple Linear Regression. In *Experimental Design and Analysis* (pp. 213–238).

Sender, R., Fuchs, S., & Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biology*, *14*(8), 1–14. https://doi.org/10.1371/journal.pbio.1002533

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., Kohli, S. K., Yadav, P., Bali, A. S., Parihar, R. D., Dar, O. I., Singh, K., Jasrotia, S., Bakshi, P., Ramakrishnan, M., Kumar, S., Bhardwaj, R., & Thukral, A. K. (2019). Worldwide pesticide usage and its impacts on ecosystem. *SN Applied Sciences*, *1*(11), 1–16. https://doi.org/10.1007/s42452-019-1485-1

Shreiner, A. B., Kao, J. Y., & Young, V. B. (2015). The gut microbiome in health and in disease. *Current Opinion in Gastroenterology*, *31*(1), 69–75. https://doi.org/10.1097/MOG.0000000000000139

Soulard, P., Dereumeaux, C., & Mercier, F. (2022). Determination of biomarkers of exposure to boscalid, captan, folpel, mancozeb and tebuconazole in urine and hair samples. *MethodsX*, *9*, 101671. https://doi.org/10.1016/j.mex.2022.101671

Stanaway, I. B., Wallace, J. C., Shojaie, A., Griffith, W. C., Hong, S., Wilder, C. S., Green, F. H., Tsai, J., Knight, M., Workman, T., Vigoren, E. M., McLean, J. S., Thompson, B., & Faustman, E. M. (2017). Human Oral Buccal Microbiomes Are Associated with Farmworker Status and Exposure. *Applied and Environmental Microbiology*, *83*(2), 1–19.

Tang, Q., Jin, G., Wang, G., Liu, T., Liu, X., & Wang, B. (2020). *Current Sampling Methods for Gut Microbiota : A Call for More Precise Devices*. *10*(April). https://doi.org/10.3389/fcimb.2020.00151

Tilg, H., Adolph, T. E., Gerner, R. R., & Moschen, A. R. (2018). The Intestinal Microbiota in Colorectal Cancer. *Cancer Cell*, *33*(6), 954–964. https://doi.org/10.1016/j.ccell.2018.03.004

Tsatsakis, A. M., Barbounis, M. G., Kavalakis, M., Kokkinakis, M., Terzi, I., & Tzatzarakis, M. N. (2010). Determination of dialkyl phosphates in human hair for the biomonitoring of Exposure to organophosphate pesticides. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *878*(17–18), 1246–1252. https://doi.org/10.1016/j.jchromb.2010.02.021

Tsuchiyama, T., Katsuhara, M., & Nakajima, M. (2017). Compensation of matrix effects in gas chromatography–mass spectrometry analysis of pesticides using a combination of matrix matching and multiple isotopically labeled internal standards. *Journal of Chromatography A*, *1524*, 233–245. https://doi.org/10.1016/j.chroma.2017.09.072

Universities of the Netherlands. (2022). *Agreement on Collective Labour Agreement for Dutch Universities (CAO-NU), 1 April 2022 to 31 March 2023 inclusive*. *April*, 1–5.

van de Wiele, T. Van de, Van den Abbeele, P., Ossieur, W., Possemiers, S., & Marzorati, M. (n.d.). Chapter

27: The Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). In *COST Action FA1005 "The Impact of Food Bio-Actives on Gut Health: In Vitro and Ex Vivo Models."* (pp. 305–318).

Van Maele-Fabry, G., Lantin, A. C., Hoet, P., & Lison, D. (2010). Childhood leukaemia and parental occupational exposure to pesticides: A systematic review and meta-analysis. *Cancer Causes and Control*, *21*(6), 787–809. https://doi.org/10.1007/s10552-010-9516-7

Vijay, A., & Valdes, A. M. (2022). Role of the gut microbiome in chronic diseases: a narrative review. *European Journal of Clinical Nutrition*, *76*, 489–501. https://doi.org/10.1038/s41430-021-00991-6

Wang, J., Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., Peng, Y., Zhang, D., Jie, Z., Wu, W., Qin, Y., Xue, W., Li, J., Han, L., … Wang, J. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*, *490*(7418), 55–60. https://doi.org/10.1038/nature11450

Wang, X., Shen, M., Zhou, J., & Jin, Y. (2019). Chlorpyrifos disturbs hepatic metabolism associated with oxidative stress and gut microbiota dysbiosis in adult zebrafish. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology*, *216*(November 2018), 19–28. https://doi.org/10.1016/j.cbpc.2018.11.010

Wang, Y., Jin, C., Wang, D., Zhou, J., Yang, G., Shao, K., Wang, Q., & Jin, Y. (2021). Effects of chlorothalonil, prochloraz and the combination on intestinal barrier function and glucolipid metabolism in the liver of mice. *Journal of Hazardous Materials*, *410*(November 2020), 124639. https://doi.org/10.1016/j.jhazmat.2020.124639

Woodruff, T. J., Kyle, A. D., & Bois, F. Y. (1994). Evaluating health risks from occupational exposure to pesticides and the regulatory response. *Environmental Health Perspectives*, *102*(12), 1088–1096. https://doi.org/10.1289/ehp.941021088

Yan, S., Tian, S., Meng, Z., Yan, J., Jia, M., Li, R., Zhou, Z., & Zhu, W. (2020). Imbalance of gut microbiota and fecal metabolites in offspring female mice induced by nitenpyram exposure during pregnancy. *Chemosphere*, *260*, 127506. https://doi.org/10.1016/j.chemosphere.2020.127506

Yang, Y. N., Yang, Y. C. S. H., Lin, I. H., Chen, Y. Y., Lin, H. Y., Wu, C. Y., Su, Y. T., Yang, Y. J., Yang, S. N., & Suen, J. L. (2019). Phthalate exposure alters gut microbiota composition and IgM vaccine response in human newborns. *Food and Chemical Toxicology*, *132*(July), 110700. https://doi.org/10.1016/j.fct.2019.110700

Zhao, Y., Zhang, Y., Wang, G., Han, R., & Xie, X. (2016). Effects of chlorpyrifos on the gut microbiome and urine metabolome in mouse (Mus musculus). *Chemosphere*, *153*, 287–293. https://doi.org/10.1016/j.chemosphere.2016.03.055

10. Appendices

10.1 Participant information sheet

PARTICIPANT INFORMATION SHEET

Study name	**Gut Microbiome Pesticide Exposure (GuMPEx)**
Contact details	Institute For Risk Assessment Sciences (IRAS) **Address**: Yalelaan 2, 3584 CM Utrecht **Phone**: +31 3025 35400 **Email**: gumpexstudy@uu.nl **Website**: http://gumpex.ac.uk/

Invitation to participate

This information leaflet is intended for potential participants for the GuMPEx study. We are inviting farmers and residents from your residential region to participate in this research. This leaflet provides you with information about the study, including its aims, what it will involve and the possible benefits and risks of participation. Please take time to read this information sheet carefully and if you would like further information, do not hesitate to contact us via the details provided. Further information about the study can be found on the study website. If you agree to participate in this study, please sign the attached consent form.

1) Why do we need this research?

We know that exposure to pesticides have adverse effects on the health of people exposed to them. The exact mechanisms of harm however are not fully understood, and it could be that the gut microbiome has a role to play in causing these diseases related to exposure. Pesticides are designed to kill undesired organisms, therefore it is likely that the microbes in the gut will be affected by exposure. Previous animal studies have shown alterations in gut microbes following experimental exposure, however we do not know the impact on the human gut microbiome.

2) What is the aim of this study?

This study aims to determine exposure of participants to commonly used pesticides and to determine whether this exposure level is associated with changes in the gut microbiome.

3) Who is conducting the study?

The study is designed and coordinated by the Institute for Risk Assessment Sciences (IRAS) at the University of Utrecht.

4) Who can participant in the study?

We are looking for non-organic and organic farmers as well as non-farming residents, all residing within the province of North-Holland. All participants must be at least 18 years. It is up to you to decide if you wish to take part or not and participation in this study is entirely voluntary.

5) What is required from me if I agree to participate?

If you decide to participate in this study, you will be required to provide faecal, urine and hair samples in both December 2023 and July 2024. In addition, you will be required to wear silicone wristbands at two time periods for a duration of 7 days. We will also request that you complete two questionnaires.

6) I would like to participate, what do I do now?

If you decide to participate, we ask now that you complete and sign the attached consent form here on the GuMPEx study database. Following this, you will receive a welcome pack in the mail along with further information of the timelines of the study.

7) What are the possible benefits of participating?

As a result of participating in the study and providing urine, wristband and hair samples for pesticide detection, we will be able to provide you with information regarding your pesticide exposure according to these measurements. In addition, we will be able to provide basic information regarding the structural composition of your gut microbiome.

8) What are the possible risks of participating?

As this study is purely observational, we do not expect any physical risks related to participation. All personal information will be pseudonymised, meaning that personally identifiable information is masked. All your personal information will be stored on a secure, encrypted database in compliance with the European Union's General Data Protection Regulation (GDPR). Only the study data manager will be able to look at the information in the study database and this information will not be passed on to anyone else. Further information regarding privacy of your information can be found on the GuMPEx study website.

9) What if I want to stop participating?

Participation is entirely voluntary and you are free to stop participating at any time throughout the study with no future implications. You are able to withdraw your consent for the use of your data throughout the study, however any information previously collected under your original consent could still be utilised in the study analysis.

10) What will happen to information about me?

All personal information will be pseudonymised and stored in the GuMPEx study database. 1 year after completion of the study (March 2026), all data from the study database will be archived in a secure data archive centre at the University of Utrecht. Valuable information regarding exposure assessment gained from this study may be of interest for future research, therefore we will ask within the consent form whether you are willing to share your exposure assessment information for future use in other research projects related to this study.

11) Do I receive any payments for participation?

All participants will receive a one-off payment of €200 for their participation in the study. If additional financial burdens arise as a result of participation, these will be reimbursed.

12) Who has reviewed this study?

This study will have been assessed by an independent committee (the medisch ethische toetsingscommissie (METC) of the Utrecht Medical Centre at Utrecht University) who will assess the risks and benefits of this proposed study for you as a participant.

10.2 Participant consent form

PARTICIPANT CONSENT FORM

Study name	**Gut Microbiome Pesticide Exposure (GuMPEx)**
Contact details	Institute For Risk Assessment Sciences (IRAS) **Address**: Yalelaan 2, 3584 CM Utrecht **Phone**: +31 3025 35400 **Email**: gumpexstudy@uu.nl **Website**: http://gumpex.ac.uk/

Please tick yes or no to the statements in the table below before signing the consent form:

1.	I have read and fully understand the Participant Information Sheet (v1.0, 01 July 2022).	Yes ☐	No ☐
2.	I have been able to ask any questions that I have regarding my participation in the study and my questions have been answered sufficiently.	Yes ☐	No ☐
3.	I understand that my participation is voluntary.	Yes ☐	No ☐
4.	I understand that I can withdraw from the study at any time.	Yes ☐	No ☐
5.	I understand that my personal information will be stored on the GuMPEx study database which will be supervised by the data manager from the study coordination team at Utrecht University.	Yes ☐	No ☐
6.	I give consent to the sharing of my pesticide exposure information with my general practitioner and the Gemeentelijke Gezondheidsdienst (GGD) in the case that my pesticide exposures exceed the acceptable daily intake.	Yes ☐	No ☐
7.	I give consent to the use of my data in future related studies.	Yes ☐	No ☐
8.	I give consent to the retention of my contact details in case the researchers would like to contact me for participation in a follow-up study.	Yes ☐	No ☐
9.	I give consent to find out my pesticide exposure levels as determined through the analysis of the samples provided in this study.	Yes ☐	No ☐
10.	I give consent for this digital consent form, which contains personal identifiers, to be stored securely on the GuMPEx study database for the duration of the study.	Yes ☐	No ☐
11.	I agree to participate in the GuMPEx study.	Yes ☐	No ☐

.....	.....	...../...../.....
Participant name	Signature	Date

** This electronic consent form will be stored in the study database and one copy will be printed and mailed to the consenting participant for their personal records.*

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