Reprogrants application december 2022.

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Institution country: Netherlands

(Optional) Gender: How do you identify? Woman

Ethnicity: White/European

Role: Post-doc

Your top 3 published papers

1. Hertzberger R, May A, Kramer G, van Vondelen I, Molenaar D, Kort R. 2022. Genetic Elements Orchestrating *Lactobacillus crispatus* Glycogen Metabolism in the Vagina. 10. Int J Mol Sci 23:5590.
2. Hertzberger R, Arents J, Dekker HL, Pridmore RD, Gysler C, Kleerebezem M, de Mattos MJ. 2014. H(2)O(2) production in species of the *Lactobacillus acidophilus* group: a central role for a novel NADH-dependent flavin reductase. Appl Env Microbiol 80:2229–39.
3. van der Veer C, Hertzberger RY, Bruisten SM, Tytgat HLP, Swanenburg J, de Kat Angelino-Bart A, Schuren F, Molenaar D, Reid G, de Vries H, Kort R. 2019. Comparative genomics of human *Lactobacillus crispatus* isolates reveals genes for glycosylation and glycogen degradation: implications for in vivo dominance of the vaginal microbiota. Microbiome 7:49.

Link to your Google Scholar profile:

https://scholar.google.com/citations?view_op=new_articles&hl=en&imq=Rosanne+Hertzberger#

Name of co-applicant(s)

Jeanine Roeters van Lennep, Monique Mulder, Remco Kort

Email of co-applicant(s)

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Institution of co-applicant

Erasmus MC, Vrije Universiteit Amsterdam

Institution country of co-applicant: Netherlands

Role of co-applicant:

Monique Mulder is biologist and associate professor at ErasmusMC. She will analyze the lipid and lipoprotein profiles using standard laboratory methods, density gradient ultracentrifugation and FPLC.

Jeanine Roeters van Lennep is an internist vascular medicine and associate professor at ErasmusMC specialized in lipid metabolism with a focus on women's health. She will provide serum samples from the biobank and vaginal samples from patients.

Remco Kort is a microbiologist and professor at the Vrije Universiteit Amsterdam and at Micropia/Artis. His research focus is planetary health and host-microbiome interactions. He will be contributing the microbial ecology perspective.

PhD Completion Year: 2013

Grant coordinator: Elma Brasser

Title (100 characters)

How premenopausal women's lipid profiles affect metabolism and colonization of vaginal bacteria

Main hypothesis and literature sources (1000 character limit).

The vaginal microbiome is associated with reproductive health outcomes. *Lactobacillus crispatus* acidifies the vagina of premenopausal women and is associated with lower risks of preterm birth and urogenital infections. The presence of fastidious anaerobes such as *Gardnerella* is associated with a higher risk (Gudnadottir et al. 2022; PMID 35562576).

Evidence has emerged that cholesterol-lowering medication in postmenopausal women is correlated with a lower abundance of *Gardnerella* (Abdelmaksoud et al. 2017; PMID 28846702). However, in premenopausal women we observed an association suggesting that higher LDL-cholesterol was associated with lower *Gardnerella* levels.

Vaginal lactobacilli require a source of fatty acids and can absorb host cholesterol (Michael et al. 2016; PMID 26839071). We hypothesize that vaginal *Lactobacillus* acidification in premenopausal women is supported by host lipids impeding colonization of bacteria with lower acid-tolerance such as *Gardnerella*.

Description of potential impact

We will study the effect of host lipids on the vaginal microbiome. To this end we will study growth of vaginal bacteria in medium with serum from women with dyslipidemias. We will also study vaginal metabolites of premenopausal women before and during use of commonly prescribed statins (cholesterol-lowering medication).

Premenopausal women increasingly use statins but the effects on reproductive health are unknown. This unique collaboration will give insight into the effects on premenopausal women and will also inform novel strategies to support vaginal *Lactobacillus* acidification with lipids.

Although all involved have previously worked on reproductive health, the internal medicine department at ErasmusMC have not previously studied the vaginal microbiome and the Vrije Universiteit have not previously experimented with women's serum or lipidomics. We expect important insights to come from the collaboration of these domains.

Technical project plan

This project consists of two phases. First, we will perform bacterial cultivation experiments using NYCIII medium prepared with serum of premenopausal female patients at the ErasmusMC outpatient lipid clinic. We will use serum from patients with various dyslipidemias, such as familial hypercholesterolemia, dysbetalipoproteinemia and hyperchylomicronemia leading to strong variations in serum lipoproteins and triglycerides.

Furthermore, we will use serum samples from women with familial hypercholesterolemia, with and without cholesterol-lowering medication (statins, PCSK9-inhibitors) and healthy controls. It has been shown previously that serum lipid levels vary according to menstrual cycle (Mumford et al. 2010; PMID 20534764). We will use serum samples of women in various stages of the natural menstrual cycle (menstrual, ovulation and luteal phase) or on oral contraceptives including a hormone-free week.

We will study growth and metabolism of various strains of common vaginal bacteria: *L. crispatus*, *L. iners, L. gasseri,* and *Gardnerella* species *(vaginalis, piotii, swidsinski, leopoldi*) and *Fannyhessea vaginalis* under strict anaerobic conditions. We will include bacterial taxa with low/no fatty acid requirements such as *Lactobacillus plantarum*. To track growth and metabolism we will measure cell density and organic acid (metabolite) concentration using HPLC.

As a control we will prepare media with controlled lipid concentrations including isolated lipoproteins (by density gradient ultracentrifugation), lipoprotein-deficient serum, free cholesterol and fatty acids (Tween80).

We will perform a screen of cholesterol, triglyceride and phospholipid levels and lipoproteins using standard laboratory methods and FPLC. This includes LDL-C, VLDL-C and HDL-C and its associated apoproteins (apoB100 and ApoA-I) in the serum-containing media before and after growth of the vaginal isolates. We will select a subset of samples for a full lipidomics screen including ~1100 lipid species using LC-ESI-MS/MS.

Previously it was shown that growth on varying lipid sources impacted stress resistance and membrane composition of lactic acid bacteria (Reitermayer et al. PMID 30001697). Changes in lactic acid tolerance are important for the ability of bacteria to colonize the premenopausal vagina. We will therefore study lactic acid tolerance using live/dead staining and flowcytometry as well as lipid membrane analysis on cell pellets grown on different lipids.

These analyses will give a full picture of species-specific differences of host lipid usage by vaginal bacteria and its effects on their physiology. These results will inform

novel vaginal lipid preparations that could steer the vaginal microbiota towards a more health-associated *L. crispatus* enriched state.

A second set of experiments will focus on the effects of cholesterol-lowering medication on vaginal *Lactobacillus* acidification in premenopausal women. We previously found premenopausal women with higher LDL-cholesterol to have lower *G. vaginalis* and *Fannyhessea vaginalis* levels (see Sup 1), that are associated with vaginal dysbiosis and adverse reproductive health. We hypothesize that this may be a reflection of higher lactate concentration, due to higher lipid availability for vaginal lactobacilli. *Gardnerella* and *Fannyhessea* are -in contrast to *Lactobacillus*- highly sensitive to lactic acid at low pH (O'Hanlon et al. 2011; PMID 21771337) and *Gardnerella* and *Fannyhessea* are associated with lower lactic acid levels (Aldunate et al. 2015; PMID 26082720).

We will study the effect of cholesterol-lowering interventions on vaginal microbiota in patients of the ErasmusMC outpatient lipid clinic who are prescribed statins or PCSK9-inhibitors for familial hypercholesterolemia. We will ask patients to provide (self-sampled) vaginal swabs before and 3 months after initiation of cholesterol-lowering medication. We will perform species-specific qPCR to quantify absolute abundance of common vaginal taxa. Furthermore we will perform metabolite analysis on HPLC to study how these medications affect vaginal microbial metabolism and acidification. We will quantify vaginal lipids to study the affect of these medications on the availability of vaginal lipids compared to serum lipids. In case our hypothesis is correct we expect a drop in lactic acid and an increase in *Gardnerella* colonization after women start using cholesterol lowering medication.

Potential pitfalls:

- We may not find an association at all between lipids and the vaginal microbiome. This is an important finding that we will publish.
- Our results may be confounded by the indirect dependency of both lipid levels and vaginal microbiome on estrogen/progesterone levels. If our results give reason to suspect this we will perform steroid hormone quantitation in blood samples. We can furthermore perform additional analyses on women at various stages of the menstrual cycle to take into account the fluctuating hormone levels.

Dollar amount requested: 100.000 USD

Brief itemization of budget (in dollars)

Salary for a technician (1 year, 0.8 fte) ~ 63.000 USD and post-doc (1 year, 0.2 fte) ~17.000 USD at the VU Amsterdam

Material budget VU Amsterdam ~ 5000 USD for Live/dead cell viability kits, chemicals, disposables, gas cilinders, additional qPCR reagents.

Budget ErasmusMC ~ 15.000 USD of which 14.000 USD for Lipidomics analysis (50 USD for 100 serum samples before and after bacterial growth. 150 USD extended lipid analysis on a subset of 60 vaginal, serum samples and bacterial cell pellets)

Patient travel costs to and from clinic, gifts and additional costs for medical-ethical committee, swabs ~1000 USD

Target timeline to completion (in months)*

12 months

By when do you think you can achieve your goal? What, if anything, would help you get there sooner? We recognize that science involves intrinsic uncertainty. We will also use this date to follow up on the progress midway.

April 2024. The first set of experiments can be performed with bacterial isolates and serum samples already available in the biobank. Delay could come from medical ethical clearance required for the second phase of experiment to receive vaginal swabs from patients at the lipid clinic and include sufficient numbers of patients including the wait time of 3 months after onset of cholesterol-lowering medication.

What resources does your institution have to help you conduct this work? (400 character limit).

ErasmusMC has a biobank with blood samples from patients with various dyslipidemias on different lipid-lowering therapy. Lipidomics facilities (LC-ESI-MSMS) will be used at the ErasmusMC and in collaboration with Prof Dieter Lütjohann (University Bonn) for serum, vaginal, and bacterial samples. The Vrije Universiteit Amsterdam has facilities for HPLC, qPCR and anaerobic cultivation.

Preliminary progress/validation on this project (600 character limit).

Previously, evidence emerged that women who take statins have a lower vaginal relative abundance of *Gardnerella* (Abdelmaksoud et al. 2017; PMID 28846702). This prompted us to study the relationship between cholesterol and vaginal microbiome in premenopausal women (see Sup. 1). Surprisingly, women with higher LDL-cholesterol had lower levels of *Gardnerella vaginalis* and *Fannyhessea vaginalis* species (associated with adverse health outcomes). In preliminary cultivation experiments studying metabolic requirements of vaginal lactobacilli we observed a strict requirement of fatty acids (Sup. 2).

Supplement 1: *Gardnerella vaginalis* and *Fannyhessea vaginalis* are significantly reduced in the vaginal microbiome of premenopausal women with high LDL-cholesterol.

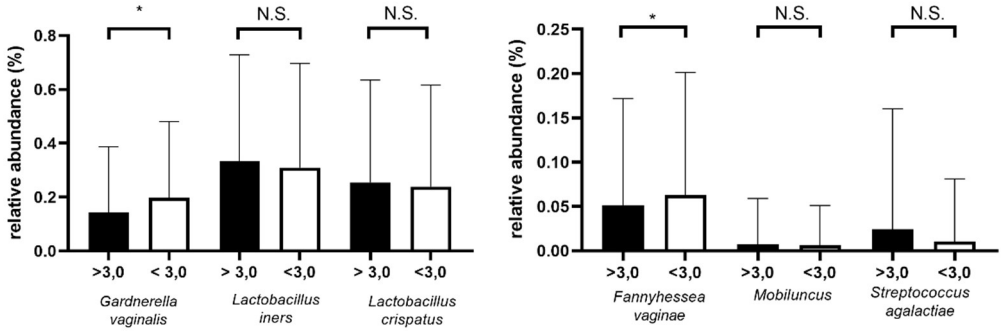

Figure 1. Abundance of six common vaginal bacterial species in the vaginal microbiome of women with normal or elevated LDL-cholesterol (<3,0 mM or >3,0 mM, respectively). We performed a secondary analysis on vaginal microbiome data in an urban cohort of Dutch premenopausal women (18-35 years old) with equal representation of each of the 6 major ethnic groups of the capital city Amsterdam (Dutch, Moroccan, Turkish, South-African Surinamese, South-Asian Surinamese and Ghanaian). Vaginal swabs were obtained from the HELIUS (Healthy Life in an Urban Setting) - database and previously published (Borgdorff et al. 2017; PMID 28700747).

We compared the relative abundance of sepcific species in 16SsRNA sequence data from vaginal swabs in women with high LDL-cholesterol compared to normal LDL-cholesterol levels. We used a cut-off of 3,0 mM to distinguish between 'high' and 'normal' consistent with the Dutch Heart Foundation definitions (Hartstichting). 147 out of 541 women had serum LDL cholesterol levels above 3,0 mM. We compared the colonization of six common vaginal species in these two groups: *Lactobacillus crispatus, Lactobacillus iners*, *Gardnerella vaginalis, Fannyhessea vaginae, Mobiluncus* and *Streptococcus agalactiae*.

Bars represent mean +/- standard deviations. We performed Mann-Whitney statistical test with * = p<0,05. We found *Gardnerella vaginalis* and *Fannyhessea vaginalis* (two species associated with vaginal dysbiosis) reduced in women with higher LDL-cholesterol.

We considered several factors that could potentially confound the association between vaginal microbiome and LDL-cholesterol: **Age**: We separately analysed *Gardnerella* levels in women older than 30 years and women older than 21 years old with LDL-cholesterol above or below 3,0 mM and in these subgroups we found the *Gardnerella* association between the two groups to still be significant (p<0,05). **BMI**: as expected women with elevated LDL-cholesterol have higher LDL-cholesterol but were found to have higher *Gardnerella* abundance on average (not significant, consistent with previous findings Brookheart et al. 2019; PMID 30707966). If BMI would be a confounding factor we would expect lower *Gardnerella* levels in the higher BMI group. **Ethnicity**: one of the groups (South-Asian Surinamese) had a higher LDL-cholesterol and lower *Gardnerella* levels. We reanalysed the cohort without this ethnic group and found the association between *Gardnerella* and LDL cholesterol to still be significantly negatively associated (p<0,05).

Supplement 2: *Lactobacillus crispatus* requires Tween80 for growth and acidification.

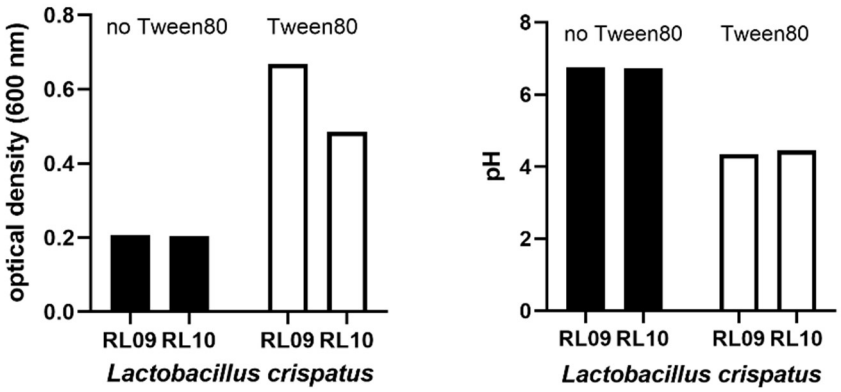

Figure 2: Growth (optical density at 600 nm) and acidification of culture media (pH) of two *Lactobacillus crispatus* strains (RL09 and RL10) after serially passaging in chemically defined medium with and without Tween80 (polysorbate, source of fatty acids). *Lactobacillus crispatus* strains were previously described (van der Veer et al. 2019; PMID 30925932) and precultured on NYCIII medium. These precultures were used to inoculate a chemically defined medium previously described (Teusink et al. 2005; PMID 16269766) with and without 1 mg/mL Tween80. After 24 hours of anaerobic incubation at 37°C, optical density and pH was measured and cultures were serially passaged by diluting 50x in fresh medium. Data are shown of passage 2 of one single experiment. The experiment has since been replicated on two independent occasions.