

**From Ultrasound to PCR:
A Modern Approach to Prenatal Pathogen Detection**

Ph.D. Thesis

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2 Publications

I.

Somogyvári, Ferenc; **Tűzkő, Nándor**; Kereszturi, Attila; Párducz, László; Szécsényi, Mária; Endrész, Valéria; Ábrók, Marianna; Ardizzone, Caleb M.; Burián, Katalin; Virok, Dezső Péter

Comparison of the inhibitory effects of Lactobacillus supernatant and coculture on Gardnerella vaginalis

BMC RESEARCH NOTES 2025;18(1):346, 7 p. (2025)

II.

Tűzkő, Nándor. ; Bartek, Virág. ; Simonyi, Atene. ; Harmath, Ágnes. ; Szabó, István. ; Virok, Dezső Péter. ; Beke, Artúr

Associations between Fetal Symptoms during Pregnancy and Neonatal Clinical Complications with Toxoplasmosis

CHILDREN (BASEL) 11 : 9, Paper: 1111, 13 p. (2024)

III.

Tűzkő, Nándor; Bartek, Virág; Beke, Artúr; Ács, Nándor

A méhen belüli toxoplasma fertőzés magzati ultrahangeltérései és a fertőzésnek kitett újszülöttek postnatalis tünetei

MAGYAR NŐORVOSOK LAPJA 88 : 2, pp. 85-94, 10 p. (2025)

3 List of abbreviations

BU	Bacteriocin unit
BV	Bacterial vaginosis
CMV	Cytomegalovirus
Ct	Threshold cycle
DNA	Deoxyribonucleic acid
HSV	Herpes simplex virus
ET	Embryo transfer
G. vaginalis	Gardnerella vaginalis
GW	Gestational week
ICSI	Intracytoplasmic sperm injection
Ig	Immunoglobulin
IUGR	Intrauterine growth restriction
IVF	In vitro fertilisation
NT	Nuchal translucency
OD₆₀₀	Optical density at 600 nanometers
PCR	Polymerase chain reaction
PTB	Preterm birth
qPCR	Quantitative polymerase chain reaction
SGA	Small for gestational age
SNI	Serious neurological injury
SUA	Single umbilical artery
TOP	Termination of pregnancy
US	Ultrasound
TORCH	Toxoplasma, Other (including syphilis and varicella), Rubella, Cytomegalovirus (CMV), and Herpes Simplex Virus (HSV)

4 Introduction

Infections during pregnancy represent a significant clinical concern due to their potential to adversely affect both maternal and fetal health. For health professionals, the understanding of complexities of these infections is essential, yet often challenging due to the wide range of pathogens involved, the varied modes of their transmission, as well as the nuanced approaches required for their diagnosis, treatment, and prevention. Pregnancy-associated infections such as those caused by *Toxoplasma gondii*, *Listeria monocytogenes*, *Cytomegalovirus*, *Rubella virus*, *Herpes simplex virus*, *Treponema pallidum*, and *Gardnerella vaginalis* can result in severe complications ranging from miscarriage and premature labor to congenital abnormalities and neonatal infections.

The TORCH panel is a crucial screening tool during pregnancy that involves tests for five infectious agents: Toxoplasma, Other (including syphilis and varicella), Rubella, Cytomegalovirus (CMV), and Herpes Simplex Virus (HSV). Detection of these infections is vital as they can cause severe congenital anomalies, fetal growth restriction, and other complications [Fitzpatrick et al, 2021]. Routine screening helps to identify pregnant women at risk and enables early interventions to mitigate adverse neonatal outcomes [Al-Hakami et al., 2020]. Despite its importance, the interpretation of results can be complex, and the diagnostic yield may vary, highlighting the need for careful consideration in clinical practice [Shqara et al., 2022].

One of the main difficulties for health care professionals lies in considering the unique immunological environment of pregnancy. The maternal immune system is modulated to tolerate the fetus, which can increase susceptibility to certain infections and alter typical immune responses.

Recent research into the modulation of the immune system during gestation has highlighted the complex interplay between immune tolerance and immune responses, which are necessary for maintaining both maternal health and fetal development (**Fig. 1**). A fundamental aspect of this process is the adaptation of the maternal immune system to accept the semi-allogeneic fetus, transitioning through various immune states of gestational stages.

During pregnancy, significant immunological changes appear, characterized primarily by a shift from a pro-inflammatory to an anti-inflammatory state. For instance, Tregoning et al noted that alterations in immune cell populations, including reductions in B cells and dendrit-

ic cells, contribute to this dynamic adaptation, enabling immune tolerance towards the developing fetus, while ensuring adequate defense against pathogens [Tregoning et al., 2020]. Similarly, Li et al identified that pregnancy leads to distinct alterations in immune responses influenced by factors such as maternal obesity, which can disrupt normal immune tolerance and fetal development [Li et al., 2021]. Martínez et al. discussed how prenatal stress can impact immune responses at the maternal-fetal interface, further complicating immune modulation during gestation [Martínez et al., 2021].

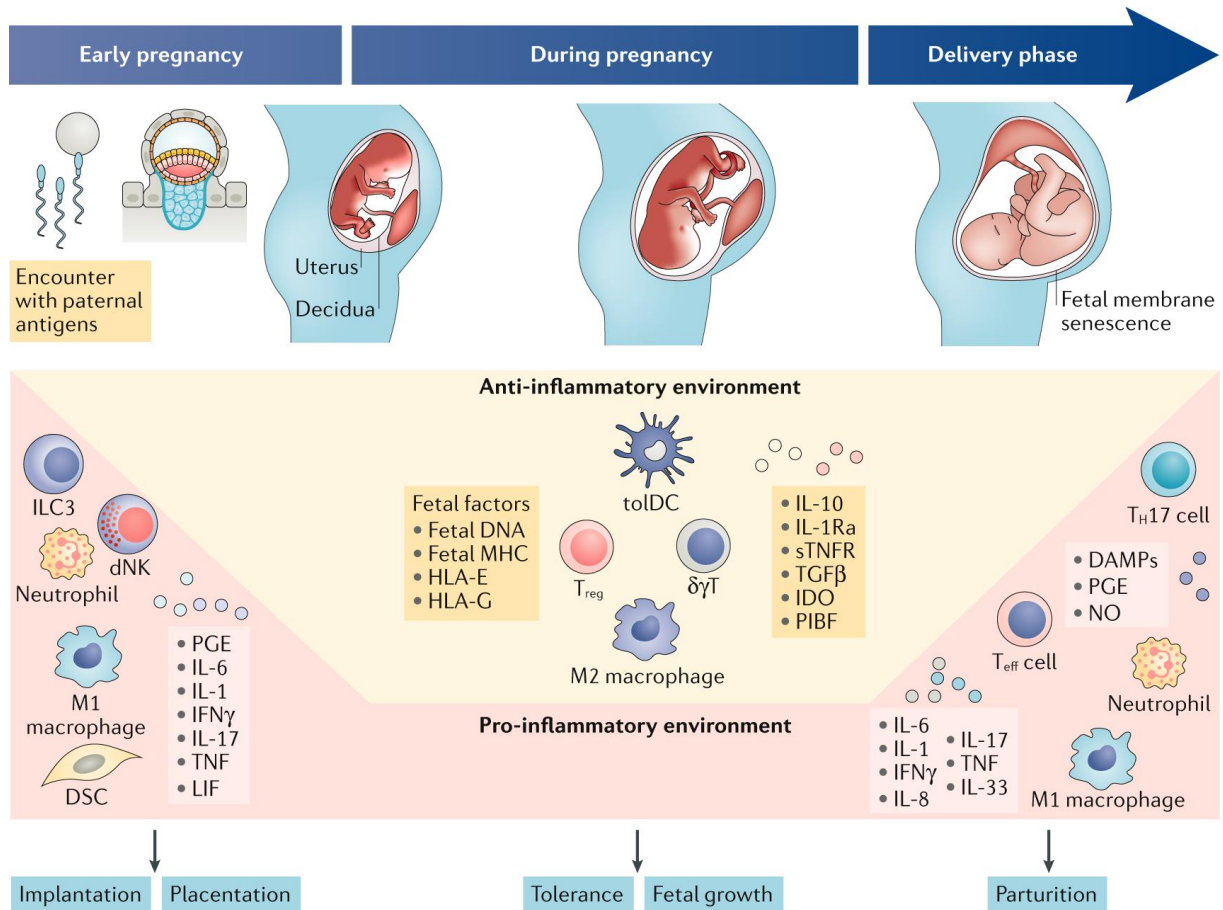


Figure 1: Immunological adaptation in pregnancy (Förger, F., Villiger, P.M. Immunological adaptations in pregnancy that modulate rheumatoid arthritis disease activity. *Nat Rev Rheumatol* 16, 113–122 (2020). <https://doi.org/10.1038/s41584-019-0351-2>)

Another crucial component of maternal immune modulation involves the role of regulatory T cells. These cells are vital for preventing maternal immune overreaction to fetal antigens, as emphasized by Chen et al., who described how regulatory T cells contribute to immune tolerance, thus reducing the risk of fetal rejection [Chen et al., 2023]. This is reflected by the

findings of Abu-Raya et al., which emphasized dynamic changes in the peripheral maternal immune system that support the necessary tolerogenic environment during normal pregnancy [Abu-Raya et al.,2020]. Moreover, Deshmukh and Way discussed how fetal T cells preferentially differentiate into regulatory T cells, a critical mechanism for averting fetal-maternal negative interactions that could jeopardize pregnancy outcomes [Deshmukh et al., 2019].

Hormonal changes during pregnancy also play a significant role in immunological adaptation. Faas et al. highlighted the influence of hormonal changes on the maternal immune response, demonstrating that similar regulatory mechanisms apply across species, including humans [Faas et al., 2023]. Hormones such as progesterone and estriol have been documented to modulate immune responses significantly, enhancing anti-inflammatory pathways that protect the fetus while preventing maternal immune overactivity [Berhan, 2020; Littauer et al., 2018]. Additionally, Hellberg discovered how pregnancy hormones may also regulate T cell immunity in conditions like multiple sclerosis, suggesting potential therapeutic models for immune regulation based on pregnancy-associated immune changes [Hellberg, 2019] (Fig.2).

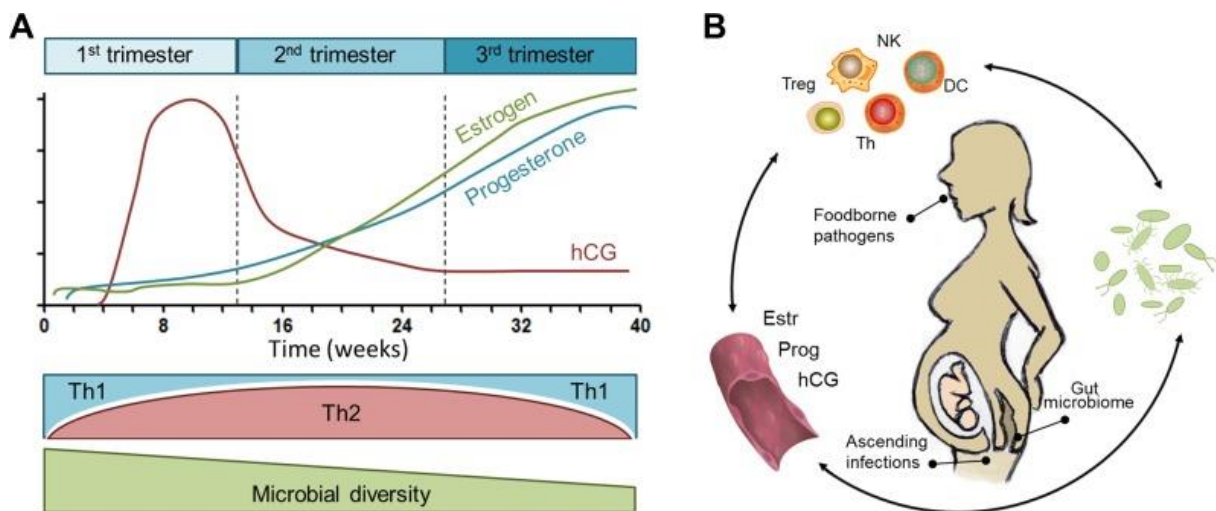


Figure 2: A. Hormonal changes during pregnancy in immunological adaptation (left side). B. Impact of the maternal gut, oral and vaginal microbiome present relationship with immunomodulation during pregnancy (right side). (<https://www.sciencedirect.com/science/article/pii/S1521691820300068>)

Besides, studies analyzing the impact of the maternal gut, oral and vaginal microbiome depict an intricate relationship with immune modulation during gestation. Wekema et al. reported that diet-induced obesity in mice alters the maternal gut microbiota and subsequently impacts immune responses at different gestational stages, indicating that microbiota compo-

sition may influence immune adaptations during pregnancy [Wekema et al., 2024]. Elderman et al. confirmed this concept by illustrating how buccal and intestinal microbiome shifts during pregnancy align with immune modifications, identifying the need for further exploration into their roles in immune tolerance and overall pregnancy health. This immunological shift means that some infections may be more severe or may be present atypically during gestation, complicating the diagnosis. Obstetricians have to learn to recognize both obvious and latent clinical signs, many of which may mimic normal pregnancy-related changes, such as fatigue, mild fever or gastrointestinal discomfort [Elderman et al., 2018].

Furthermore, the idea of vertical transmission — where pathogens are passed from mother to fetus via the placenta, during childbirth, or via breastfeeding — adds another degree of complexity. For instance, *Toxoplasma gondii*, a protozoan parasite, can cause devastating effects on the fetal central nervous system if transmitted in utero [Shiono et al., 2007]. Similarly, *Cytomegalovirus* and *Herpes simplex virus* can result in hearing loss, developmental delays, or even neonatal death. The timing of maternal infection is related to gestational age, as this significantly influences the risk and severity of fetal outcomes [Flegr et al., 2014, Wekema et al., 2024]. The diagnosis and treatment of pregnancy-related infections are also complicated by the need of both maternal and fetal protection [Shiono et al., 2007]. The test interpretation is difficult in some cases, because of the high avidity in pregnancy. The test interpretation are in **Figure 3 and 4**.

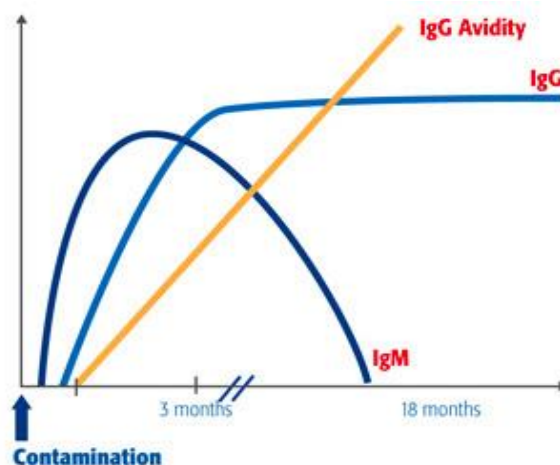


Figure 3. Binding affinity and avidity of IgG and IgM antibodies after an infection. (Phadke: How to interpret TORCH investigations. <https://www.linkedin.com/pulse/how-interpret-torch-investigations-short-guide-ajay-phadke/> updated: 13 Aug 2017)

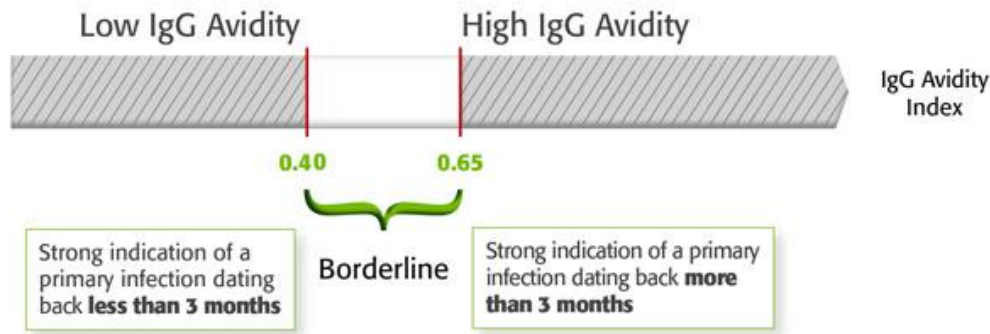


Figure 4. Avidity shifts from low to high level after about 3 months. If the avidity is high, this suggests infection occurred at least 3 months before testing. (Phadke: How to interpret TORCH investigations. <https://www.linkedin.com/pulse/how-interpret-torch-investigations-short-guide-ajay-phadke/> updated: 13 Aug 2017)

A lot of antimicrobial agents commonly used in non-pregnant individuals are contraindicated during pregnancy due to teratogenic effects. As a result, we must become familiar with safer alternatives and weigh the risks and benefits of therapeutic interventions. This requires a team work of pharmacologists, microbiologists and obstetricians [Arora et al., 2017].

Preventive strategies, including vaccination, dietary precautions, and screening protocols, are another essential tools for healthcare professionals [Tregoning et al., 2020]. For example, avoiding undercooked meat and contact with cat faeces is crucial in preventing toxoplasmosis, while regular prenatal screening can help to identify infections like syphilis, HIV, and bacterial vaginosis early in pregnancy. Training patients effectively is a key indicator for future clinicians, making communication training equally important in medical education [Shiono et al., 2007, Guerina et al., 1994, Belanger et al., 2025].

Infections in pregnant women demonstrate a multifaceted challenge for maternal care, combining elements of infectious disease, obstetrics, immunology, and pharmacology [Belanger et al., 2025]. Mastering this topic is critical not only for academic success, but also for future clinical practice, where early recognition and appropriate management of infections can profoundly influence maternal and neonatal outcomes [Liu et al., 2020; Fitzpatrick et al., 2022].

Pregnancy represents a unique immunological state that increases susceptibility to certain infections, which can have significant implications for both maternal and fetal health [Flegr et al., 2014; Wekema et al., 2024]. Among these, *Toxoplasma gondii* and *Gardnerella vaginalis* are two important pathogens that are associated with adverse pregnancy outcomes [Liu et al 2020, Fitzpatrick et al., 2022].

Understanding the impact and mechanisms of these infections during pregnancy is essential for early diagnosis, prevention, and therapeutic intervention to reduce the risk of vertical transmission and ensure optimal maternal and fetal health.

4.1 Toxoplasmosis

Toxoplasmosis is the most common parasitism in the world [Flegr et al., 2014; Furtado et al., 2011], with some estimates suggesting that 60% of the population are carriers [CDC 2024]. Researchers assume that at least one third of the population has been infected with *Toxoplasma gondii* during their lifetime [Fallahi et al., 2018]. Humans can acquire toxoplasmosis through several ways: eating undercooked meat containing the parasite, contact with cat faeces, via maternal circulation to fetus during pregnancy, organ transplantation or blood transfusion. Based on estimates, 190,000 new cases of congenital toxoplasmosis can be recognized globally every year [Torgerson 2013]. Among the statistical data of the European Center for Disease Prevention and Control for 2021, 150 cases of congenital toxoplasmosis are listed in European countries [ECDC 2024].

Toxoplasma gondii, an obligate intracellular parasite, is the causative agent of toxoplasmosis and a member of the TORCH complex—a group of pathogens known to cause congenital infections [Voekt et al., 2017]. Although toxoplasmosis is often asymptomatic in immunocompetent individuals, during pregnancy it can lead to severe fetal complications such as hydrocephalus, intracranial calcifications, chorioretinitis, and even miscarriage [Belanger et al., 2025, Kochanowsky et al., 2018]. The risk of fetal transmission increases with gestational age, but the severity of outcomes is usually greater when infection occurs earlier in pregnancy.

Pregnant women may contract toxoplasmosis in two ways. More commonly, this takes place through contact with infected oocysts (consumption of undercooked meat or contact with cat litter) [Gilbert et al., 2002, Kochanowsky et al., 2018, Jones et al., 2008] (**Fig. 5**).

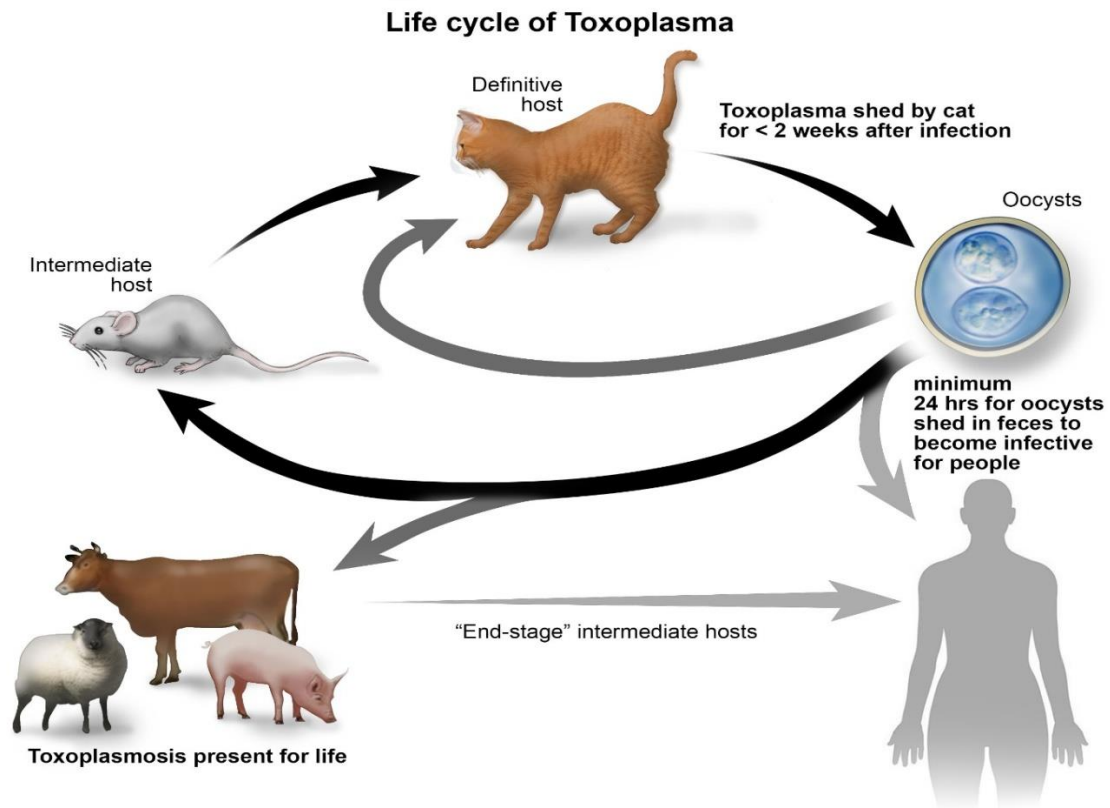


Figure 5: Life cycle of *Toxoplasma gondii*. (<https://www.vet.cornell.edu/departments-centers-and-institutes/cornell-feline-health-center/health-information/feline-health-topics/toxoplasmosis-cats>)

Toxoplasma gondii can reach the decidua via maternal immune cells and leukocytes, or in the decidua itself, entering the immune cells and afterwards the extravillous cytotrophoblasts [Arora et al., 2017, Shiono et al., 2007] (Fig. 6).



Figure 6. *Toxoplasma gondii*, shown here in one of its asexual phases (<https://www.science.org/content/article/no-kittens-required-scientists-find-new-way-study-toxoplasmosis-parasite-lab>)

Toxoplasma infection can be detected by both direct and indirect methods. The most common indirect method is immunoglobulin detection from maternal blood (IgM) [Marquez-Mauricio et al., 2023]. PCR testing of amniotic fluid can be utilized to confirm vertical infection, but is rather expensive for screening. The first protocol describing the qualitative detection of *Toxoplasma gondii* DNA by PCR dates back to 1989. Burg et al. sequenced and amplified the B1 region of the parasite, which is repeated 35 times in the parasite genome [Burg et al., 1989]. Since then, other target regions could be sequenced using local protocols, and thus, quantitative measurements can be performed [Galvani et al., 2019].

Timely diagnosis and treatment are essential to prevent serious complications [Bollani et al., 2022]. The severity of congenital toxoplasmosis depends on the week of gestation and whether the pregnant woman has had the infection before [Weiss et al., 2009].

A treated Toxoplasma infection reduces the chance of both prenatal and postnatal complications. As in all cases, Toxoplasma infection associated with preterm birth carries a higher risk of subsequent complications. All children with known congenital Toxoplasma infection require close ophthalmologic monitoring until early adulthood [Kota et al., 2023]. The most common intrauterine fetal malformations are those affecting the nervous system (macrocephaly, hydrocephalus and neural calcification), small for gestational age/intrauterine growth restriction (SGA/IUGR) and hepatosplenomegaly [McAuley et al., 2014, Wallon et al., 2004] (**Fig. 7**).

Ultrasound signs that may alert to intrauterine infection include intracranial calcification, ventriculomegaly (**Fig. 8**), hydrocephalus, hepatosplenomegaly with or without calcification involving the gastrointestinal system, and hyperechogenic bowels (**Fig. 9**). In addition, ascites, pericardial effusion and hydrops or polyhydramnios may be present. SGA/IUGR is also frequently observed [Saso et al., 2020].

The Sabin-Pinkerton triad in classic congenital toxoplasmosis comprising chorioretinitis, hydrocephalus and neurological calcification can be diagnosed in cases where the pregnant woman acquires the primary infection predominantly in the first trimester and receives no further treatment [Belanger et al., 2025, Kota et al., 2023]. However, in 85% of the cases, no abnormality is can be determined by routine neonatal investigations [Wallon et al., 2004].

In a study 40% of the confirmed cases of intrauterine infection were regarded to be free of abnormalities at routine neonatal examination, but later ophthalmological or neurological ab-

normalities became apparent. Therefore, early diagnosis of these subclinical abnormalities could reduce the extent of subsequent damage [Guerina et al. 1994].

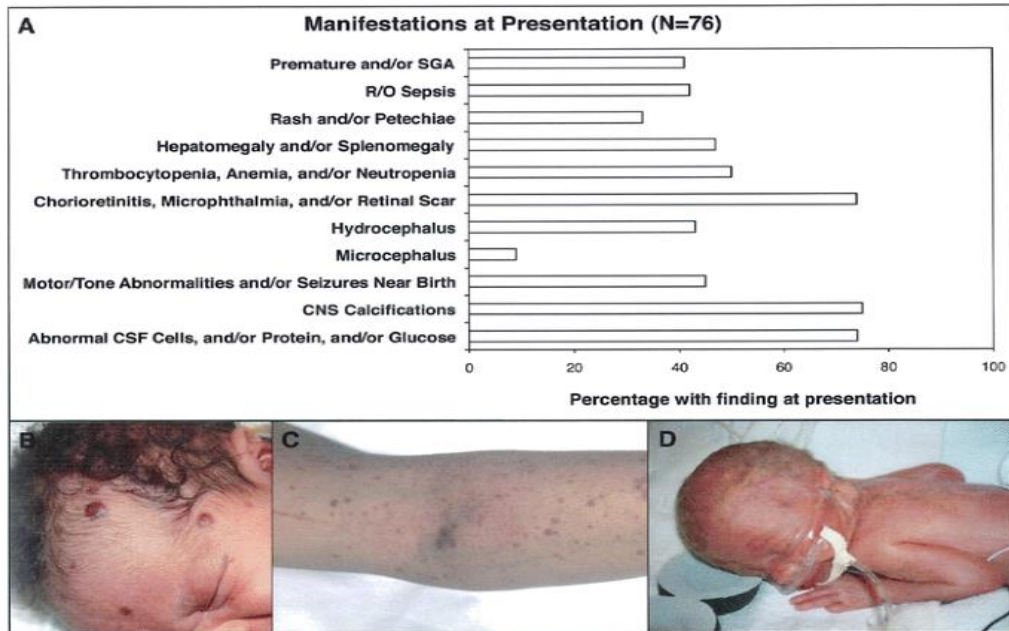


Figure 7: Intrauterine manifestation of toxoplasmosis. (A) Percentage of patients with specific manifestations of congenital Toxoplasma gondii infection. (B) Blueberry muffin rash as the skin manifestation in congenital toxoplasmosis. (C) Petechiae secondary to thrombocytopenia. (D) Prematurity, as a result of Toxoplasma gondii infection. (McLeod, R., Lykins, J., Gwendolyn Noble, A. et al. Management of Congenital Toxoplasmosis. Curr Pediatr Rep 2, 166–194 (2014). <https://doi.org/10.1007/s40124-014-0055-7>)



Figure 8. The normal brain (left) and ventriculomegaly (right) in fetal ultrasound images uncovered by transabdominal investigation in the second trimester (own images).

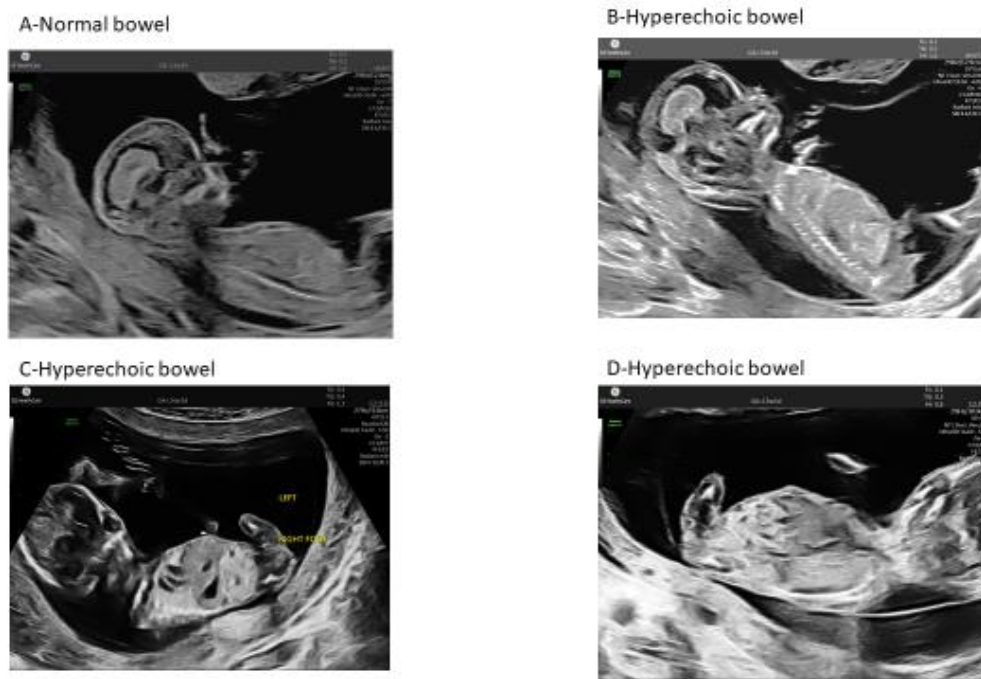


Figure 9. The normal and hyperechogenic bowel are demonstrated in fetal ultrasound images after transabdominal examination in the first trimester (own images).

Accordingly, neonatal and late complications most frequently affect the nervous system, and the proportion of children with special educational needs and late speech and motor development is higher in this group. Ophthalmological complications, early visual as well as hearing impairment can also be manifested. Toxoplasma induced chorioretinitis affects approximately 21,000 people worldwide each year [Deganich et al., 2022]. Neonatal follow-up should be systematic and involve co-specialists. Blood samples should be sent for serological testing from both the newborn and the mother as soon as possible after birth. IgM and IgA levels of the newborn baby should be checked at the age of 3 months and IgG levels by the age of 1 year to screen for late seroconversion. By the age of 1 year, all maternally derived Toxoplasma-specific IgG can disappear in the infant. In addition to serological tests, the complete blood count as well as the values of liver and kidney function should also be scrutinized. The neonate should also be referred to a pediatric ophthalmologist, and lifelong ophthalmic follow-up is recommended for the affected patients. The more frequent hearing tests are also advised. For newborns with organ damage, treatment should be started as soon as possible. There is currently no clear standard of care, but in most countries, pyrimethamine, sulfadiazine and folic acid are recommended in combination up to 1 year of age. Both treated and

suspected cases of infection should be kept under close neonatal and then pediatric surveillance [Saso et al., 2020].

4.2 Gardnerella infection

Gardnerella vaginalis (*G.vaginalis*) is a facultative anaerobic bacterium which is commonly associated with bacterial vaginosis (BV), a condition characterized by the disruption of normal vaginal flora, particularly the depletion of the protective *Lactobacillus* species.

BV is a common condition during pregnancy that can lead to severe complications if left untreated. BV is characterized by a disruption of normal vaginal microbiota, resulting in a decrease in *Lactobacillus* species and an overgrowth of various pathogenic bacteria, including *Gardnerella vaginalis*, which can trigger a cascade of adverse pregnancy outcomes [Wang et al., 2025]. Studies indicate that the presence of *Gardnerella vaginalis* increases the risks of preterm birth (PTB), late miscarriage, and preterm premature rupture of membranes, underscoring its clinical significance in obstetrics [Wang X et al, 2025; Tajadura-Ortega et al 2025]. The presence of *G. vaginalis* during gestation (especially in the context of dysbiosis) poses a risk to both maternal reproductive health and neonatal outcomes.

The pathogenesis of *G. vaginalis* in BV involves the production of virulence factors, such as sialidase and vaginolysin, which are associated with the ability to invade the vaginal epithelium and form biofilms. These virulence characteristics complicate the effective treatment, contributing to recurrence and difficulties in management [Peebles et al., 2019; Tajadura-Ortega et al 2025]. Failed treatments are often attributed to the presence of metronidazole-resistant strains of *Gardnerella*, highlighting the need for comprehensive treatment strategies [Wang X et al, 2025; Tajadura-Ortega et al 2025]. Moreover, the immune response in pregnant women may be compromised during BV, further exacerbating the risk of intrauterine infections. The complex interactions between hormonal changes during pregnancy and the immune modulation associated with *G. vaginalis* suggest that a multifaceted approach is necessary for effectively managing BV to reduce potential complications [Wang X et al, 2025; Tajadura-Ortega et al 2025]. Given the high prevalence of BV in pregnant women and its links to adverse outcomes, routine screening and appropriate treatment are crucial components of antenatal care [Wang X et al, 2025; Tajadura-Ortega et al 2025, Peebles et al., 2019].

Lactobacillus species play a crucial role in maintaining healthy vaginal microbiota by producing lactic acid, hydrogen peroxide, and bacteriocins, inhibiting the overgrowth of pathogenic

bacteria [Chee et al., 2020] (**Fig. 10**). A decrease in *Lactobacillus* abundance is a hallmark of BV, where the protective lactobacilli are outnumbered by other bacteria in the vaginal flora. The socioeconomic impact of BV is significant, with a prevalence ranging from 23% to 29% in the general population [Peebles et al., 2019].

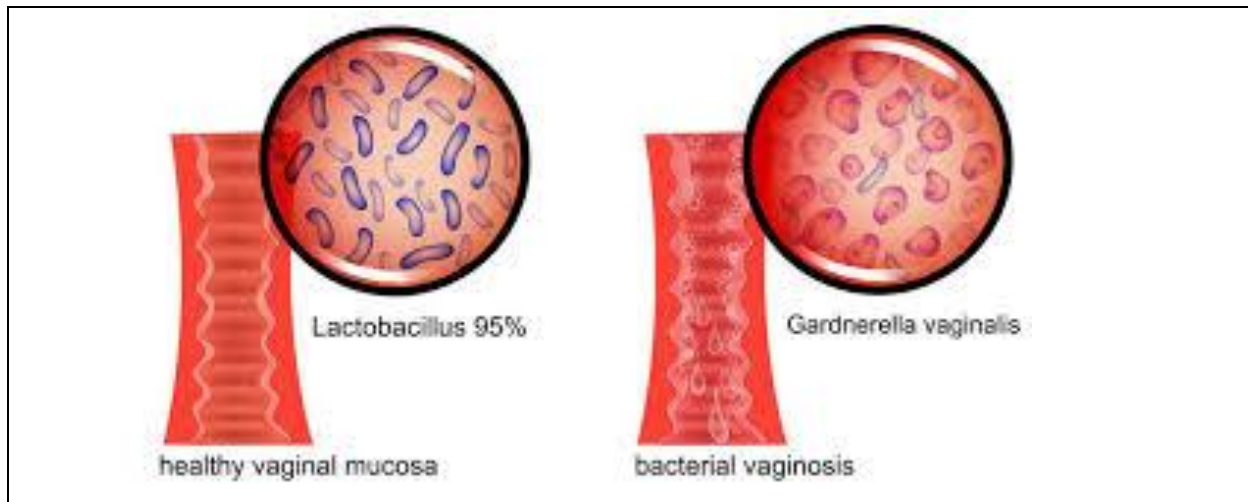


Figure 10. Role of *Lactobacillus* species in maintaining healthy vaginal microbiota. Source: <https://dubaistdclinic.com/gardnerella-vaginalis.html>

G. vaginalis, a Gram-variable bacterium known as a key player in the pathogenesis of BV, is often found in high abundance in affected women [Chen et al., 2021]. *Lactobacillus* can effectively inhibit *G. vaginalis*, and the presence of *Lactobacillus* species is often inversely correlated with the abundance of *G. vaginalis* in vaginal samples [Bae et al., 2024].

Screening the antimicrobial activity of vaginal probiotic candidates against relevant microorganisms, including *G. vaginalis*, is a crucial step in the selection process. A common method involves testing the cell-free supernatants of Lactobacilli in liquid cultures of pathogens, such as *G. vaginalis* [Pessoa et al., 2017]. While this method is relatively simple, it fails to replicate the natural interactions between Lactobacilli and *G. vaginalis* that exist *in vivo* within a shared microenvironment. Coculture methods, in which *Lactobacillus* strains are incubated with *G. vaginalis*, can address this issue. A challenge with these approaches is the selective measurement of *G. vaginalis* growth against *Lactobacillus* background. For example, an agar method was developed in which *Lactobacillus* isolates were cultivated on MRS agar and overlaid with Mueller-Hinton agar containing the pathogenic bacterium [Ahire 2023]. The zone of inhibition served as a measure of antimicrobial activity. Although these methods are well-established, they require considerable manual work, making them less suitable for screening.

5 Aims of studies

5.1 Toxoplasmosis

The aim of our study was to investigate which ultrasound abnormalities are most commonly associated with active Toxoplasma infection and the long-term complications of the infection. Based on the retrieved literature, we designed a prospective prenatal ultrasound study. Our aim was to investigate whether there was a correlation between expected complications and prenatal ultrasound alterations.

5.2 Gardnerella infection

Screening the antimicrobial activity of vaginal probiotic candidates against relevant microorganisms, including *G. vaginalis*, is a crucial step in the selection process.

Our goal was to develop a cost-effective, rapid, reproducible screening method that requires slight manual labour.

6 Hypotesis

6.1 Toxoplasmosis

1. The review of literature and our prenatal ultrasound investigation can present a sensitive and specific ultrasound marker for detecting active Toxoplasma infection.
2. The fetal ultrasound alterations will indicate later neonatal complications. This allows us to predict the appearance and extent of neonatal infectious complications by means of a non-invasive method.

6.2 Gardnerella infection

1. The direct qPCR will be effective for measuring the antimicrobial activity, as a screening method in bacterial vaginosis.
2. Cocultures that may reflect to *in vivo* microbial interactions better should also be used to evaluate *Lactobacillus*-mediated inhibition of *G. vaginalis* growth.

7 Material and methods

7.1 Toxoplasmosis

In our prospective study, we analyzed cases of recent maternal *Toxoplasma* infections confirmed by serological testing at the Department of Obstetrics and Gynecology, Semmelweis University, Hungary, between 1996 and 2020. We included in the study those pregnant women who applied for genetic counselling at the department and who were confirmed to be infected with *Toxoplasma* by serological testing and who requested amniocentesis. Those who had a miscarriage before amniocentesis or did not request amniocentesis were excluded from the study.

7.1.1 Serological test

The serological tests (IgG and IgM determination) were performed at the Central Laboratory of Semmelweis University in accordance with international recommendations, and IgA determination was also performed in cases of acute infection. In recent infections detected during serological testing, genetic counseling was provided, where the couple was informed in detail and additional ultrasound examinations were performed, if necessary and amniocentesis was offered to detect fetal involvement.

7.1.2 Ultrasound examinations

7.1.2.1 Review of literature

All scientific articles available via PubMed and Embase published after 1995 and matching the search terms “toxoplasmosis”, “ultrasound”, “prenatal” and “infant” were screened. After a detailed analysis of the abstracts, we excluded those that were not written in English, summary studies, meta-analyses, experimental descriptions, or descriptions in animal models, as well as articles that were not available through PubMed or Embase. In processing the abstracts, we excluded all articles that did not provide an adequate answer to our research question, i.e., which prenatal ultrasound abnormalities are associated with congenital toxoplasmosis. We excluded from processing all articles where toxoplasmosis and other TORCH infections were described collectively. The process was carried out using Rayyan software. **..Figure 11** summarizes the decision process.

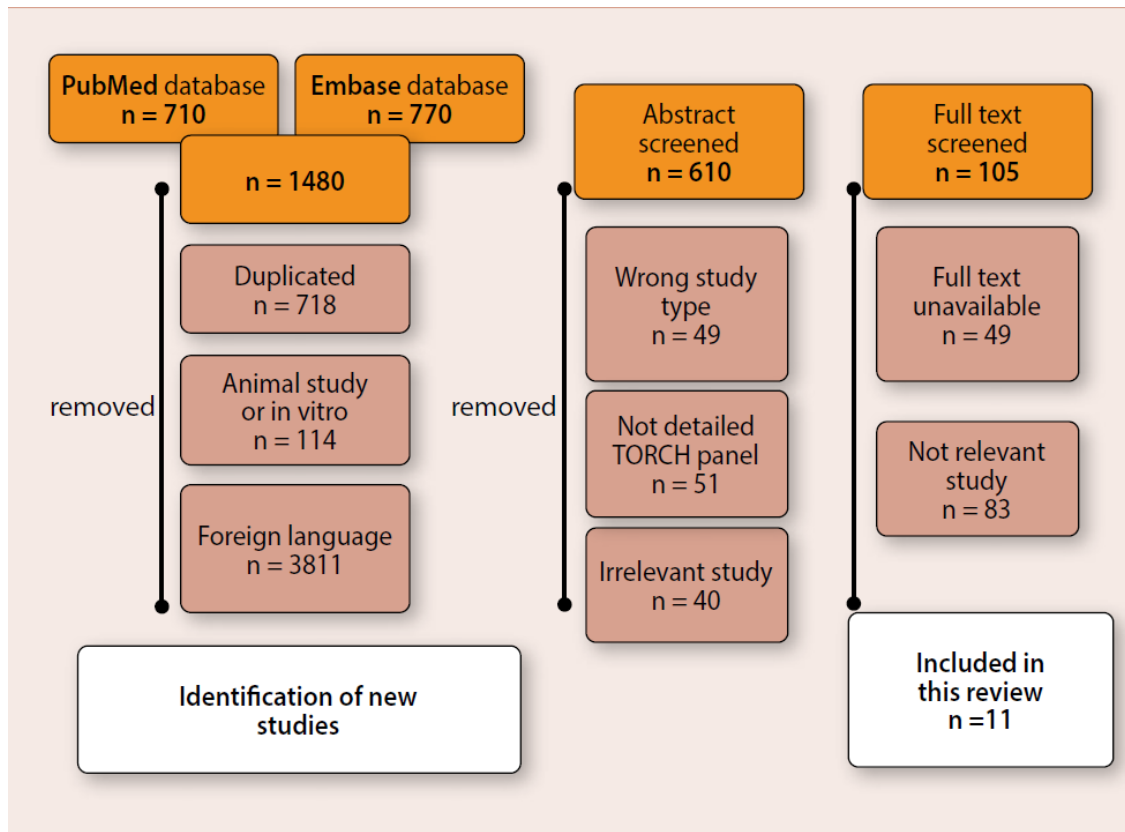


Figure 11: *The algorithm that we used for the systematic literature review of prenatal and postnatal analysis of toxoplasmosis*

7.1.2.2 Own ultrasound investigations

Ultrasound examinations were performed during the second trimester (19-22 gestational weeks) at the Ultrasound Laboratory of the Department of Obstetrics and Gynecology, Semmelweis University, Hungary.

The ultrasound equipments used in the study were as follows: Medison Sonoace X8 (Samsung Medison Co. Ltd., Seoul, Republic of Korea); Samsung Medison UGEO H60 (Samsung Medison Co. Ltd., Seoul, Republic of Korea); Samsung Medison WS80A (Samsung Medison Co. Ltd., Seoul, Republic of Korea); Philips® HD 11XE (Philips Ultrasound, Amsterdam, The Netherlands). The ultrasound equipments were fitted with 2-6 MHz transabdominal convex transducers. The examinations were carried out in accordance with the professional protocols developed by the Hungarian Society of Ultrasound in Obstetrics and Gynecology (Obstetrical transabdominal ultrasound examination; Fetal echocardiography; Ultrasound examinations recommended during pregnancy issued on 10 February 2003) and the current Hungarian guidelines for healthcare professionals (On the diagnostical and basic ultrasound screening examinations in early pregnancy, 2016).

As for current guidelines significant fetal anomalies include fetal cranial abnormalities (ventriculomegaly, cerebral calcification, ventricular dilatation III and IV and microcephaly), subcutaneous edema (nuchal translucency (NT), hydrops, anasarca and hygroma), cardiac and thoracic abnormalities (pericardial effusion, pleural effusion and thoracostenosis), abdominal anomalies (hyperechogenic bowels, distended bowels, hyperechogenic liver, perihepatic fluid, ascites and pyelectasia) as well as placental anomalies (cystic placental abruptions, thickened placenta, premature calcification and amniotic bands). In cases of ultrasound signs suggestive of an infection, genetic counselling was provided, during which the expectant mother/couple was informed in detail about the possible risks and complications of the infection.

7.1.3 Amniocentesis

If the serological result was positive or inconclusive, or if a new or worsening lesion was seen on a check-up ultrasound examination, amniocentesis was recommended to detect possible intrauterine transmission. The examinations were performed in the second trimester, in parallel with ultrasound investigations.

7.1.4 DNA isolation

After amniocentesis the amniotic fluid sample was stored at 4–8 °C, and after 1–2 h we proceeded with DNA isolation. After DNA isolation we stored the samples at –80 °C (**Fig. 12**). DNA isolation from amniotic fluid was completed at the Genetics Laboratory of the Department of Obstetrics and Genetics, Semmelweis University, using silica gel adsorption technique (High Pure PCR Template Isolation Kit, Roche, Mannheim, Germany). As positive control, *Toxoplasma gondii* (RH strain) DNA was extracted from purified parasites originating from the ascites fluid of intraperitoneally inoculated mice. Tenfold dilution with distilled water was applied to obtain DNA concentrations corresponding to 10⁸–10² parasites.

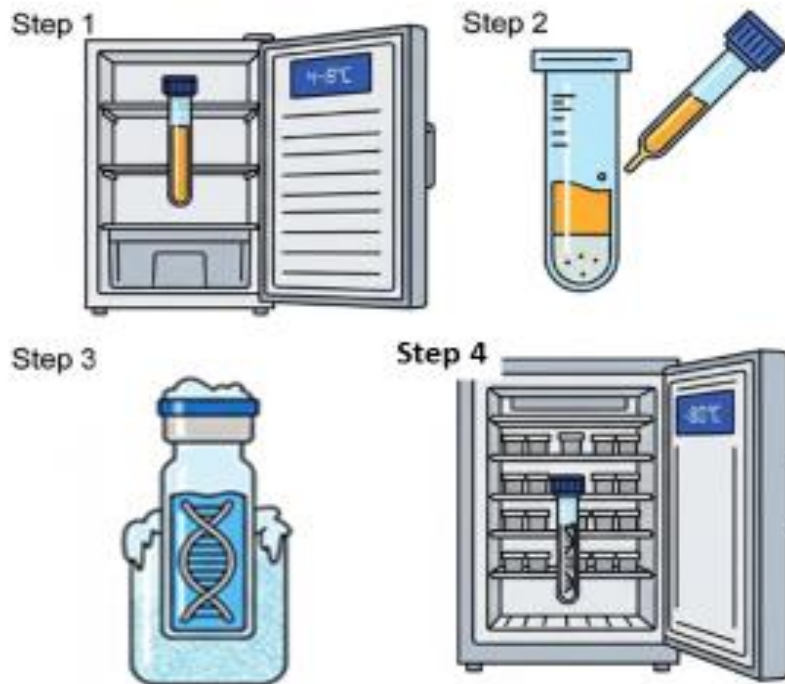


Figure 12. Sample handling before analyzing *Toxoplasma gondii*. (N. Túzkó created by MindThe Graph)

7.1.5 Fluorescent PCR and DNA fragment analysis

Fluorescent PCR and DNA fragment analysis were used for the assay. For amplification, a 1 μ L DNA sample was handled. Toxo B22, TET-5'-AAC GGG CGA GTA GCA CCT GAG GAG A-3' and Toxo B23, 5'-TGG GTC TAC GTC GAT GGC ATG ACA AC-3' primers were implemented. The PCR reaction mixture contained 2.5 μ L PCR buffer, 2.5 μ L 10 mM dNTP, 2.5 μ L 25 mM MgCl₂ and 0.15 U AmpliTaq Gold DNA Polymerase in a final volume of 25 μ L. The initial incubation lasted for 10 min at 95 °C, followed by denaturation (95 °C, 30 s), tempering (55 °C, 45 s), extension (72 °C, 45 s) for 36 cycles and finally by an incubation at 72 °C for 30 min. Four microliters of F-PCR product were mixed with 20 microliters of formamide (Sigma-Aldrich, St. Louis, MO, USA) and one microliter of Prism GeneScan-500 TAMRA internal standard (PE Applied Biosystems, Warrington, UK). The mixture was then denatured at 95 °C for 3 minutes and cooled to 4 °C for 5 minutes. Electrophoresis was conducted with the help of an ABI 310 Genetic Analyser using POP4 gel (PE Applied Biosys-

tems, Foster City, CA, USA). The results were analyzed using GeneScan Analysis software 3.1 (PE) (**Fig. 13**).

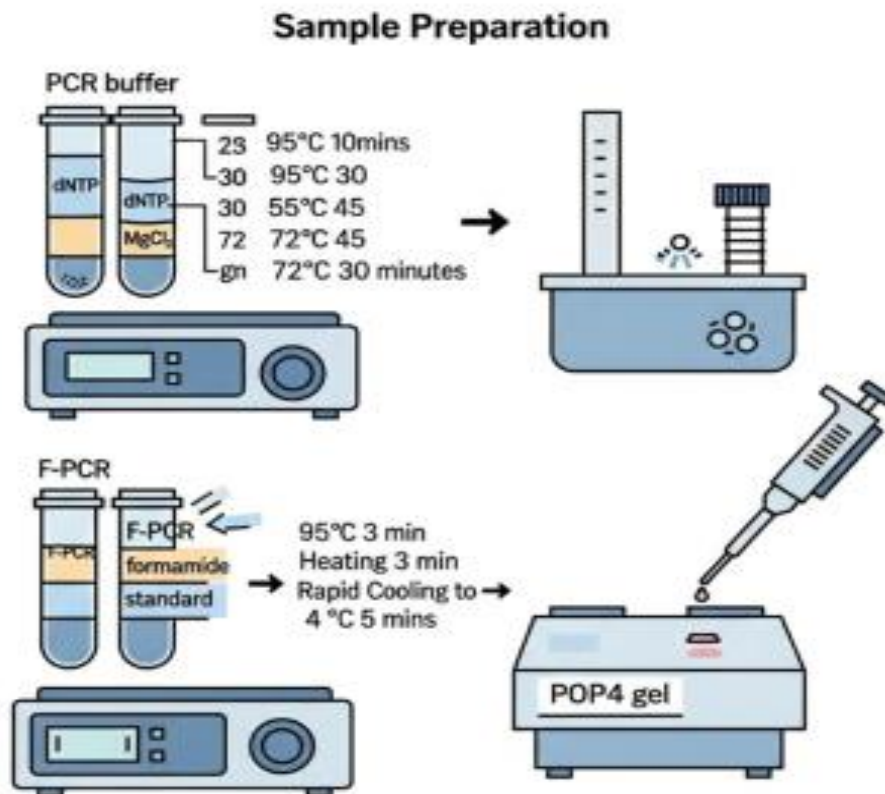


Figure 13: Sample handling for DNA fragment analysis. (N. Túzkó created by MindThe Graph)

7.1.6 Postnatal follow-up

After birth, a neonatological, microbiological, pediatric neurological and ophthalmological examination was accomplished and follow-up was carried out. In the postnatal period, after the maternal and newborn serological examination, a cranial ultrasound examination was performed and an ophthalmological examination was fulfilled. In positive cases, medication was administered. Children with Toxoplasma infection underwent regular neurological and ophthalmological control, respectively follow-up.

7.2 Gardnerella infection

7.2.1 Bacterial strains

Two *Lactobacillus crispatus* (*L. crispatus*-200, *L. crispatus*-202), two *Lactobacillus gasseri* (*L. gasseri*-212, *L. gasseri*-224), one *Lactobacillus jensenii* (*L. jensenii*-241), and one *G. vaginalis* isolate were gained during routine microbiological diagnostic procedure of vaginal swab samples (Department of Medical Microbiology, University of Szeged, Szeged, Hungary) and were used in this study. Species-level identification was executed using MALDI Biotyper (Bruker, Billerica, MA, USA).

7.2.2 Growth kinetics of *G. Vaginalis* and *Lactobacillus* strains

Lactobacillus isolates (n=4) and *G. vaginalis* (n=4) were cultured in either 200 µl MRS medium (Bio-Rad, Hercules, CA, USA) or NYC-III medium (ATCC Medium 1685) for 72 h at 37°C with 5% CO₂. The initial concentrations were adjusted to 0.1 OD₆₀₀. OD₆₀₀ was measured at 0, 2, 4, 6, 8, 24, 48 and 72 h (*G. vaginalis*) or 0, 24, 48 and 72 h (*Lactobacillus* spp.) using an EZ Read 400 Microplate Reader (Harvard Bioscience, Holliston, MA, USA).

7.2.3 Direct qPCR of *Gardnerella vaginalis*

Direct qPCR of *G. vaginalis* was conducted with the use of HOT FIREPol EvaGreen qPCR Supermix (Solis Biodyne, Tartu, Estonia) in a Bio-Rad CFX96 real-time PCR System (Bio-Rad, Hercules, CA, USA). The initial qPCR experiments utilized four primer pairs previously documented in the literature and were named Primer-1, Primer-2, Primer-3 and Primer-4. Following a preliminary sensitivity screen, the primer pairs (Primer-3) from Zozaya-Hinchliffe et al. were selected (Zozaya-Hinchliffe et al. 2010) with the primer sequences 5'-GGAAACGGGTGGTAATGCTGG-3' and 5'-CGAAGCCTAGGTGGGCCATT-3'. The qPCR mixture contained 2 µl HOT FIREPol EvaGreen qPCR Supermix, 1 µl forward and reverse primers (10 pmol each), 1 µl template, and 5 µl Milli-Q water, totalling 10 µl. The qPCR started with a 12 min activation step at 95°C and was followed by 40 cycles at 95°C for 15 sec, at 68°C for 25 sec, and at 72°C for 20 sec, after which fluorescence was measured. Genomic DNA from *G. vaginalis* was extracted using the Zymo Research Quick-DNA Mini-prep kit (Zymo Research, Irvine, CA, USA) and used as a comparison.

7.2.4 Inhibition of *G. vaginalis* growth by *Lactobacillus* supernatant

10 µl of *Lactobacilli* adjusted to 0.1 OD₆₀₀ were inoculated into 1 ml of NYC-III medium and incubated for 48 h at 37°C with 5% CO₂. After incubation, the supernatants were obtained by harvesting the cells through centrifugation for 15 min at 8,000×g and were filtered using 0.22 µm Millex-GS Filter Units (Sigma-Aldrich, St. Louis, MO, USA). *G. vaginalis* (0.1 OD₆₀₀) was cultured in NYC-III medium with 50%, 25%, and 12.5% v/v *Lactobacillus* cell-free supernatants for 48 h at 37°C with 5% CO₂. OD₆₀₀ was measured at 48 h (n=3).

7.2.5 Inhibition of *G. vaginalis* growth by *Lactobacillus* coculture

Lactobacillus isolates and *G. vaginalis* were propagated separately in NYC-III medium for 24 h at 37°C with 5% CO₂. Initial OD₆₀₀ values were adjusted to 0.1 or 0.01. *Lactobacillus* isolates were mixed with *G. vaginalis* at ratios of 10:1, 1:1 and 1:10. *Lactobacillus* and *G. vaginalis* cocultures were incubated for 48 h at 37°C with 5% CO₂. Direct qPCR was utilized to quantify *G. vaginalis* concentrations in the culture media (n=4).

7.3 Statistical methods

The differences in categorical variables were assessed using Fisher's exact test. Statistical analysis was managed using Stata Statistical Software (version 13.0, StataCorp, College Station, TX, USA), with a statistical significance set at $p < 0.05$.

The statistical comparisons of *G. vaginalis* samples were surveyed using one-way ANOVA and Student's t-test, with a significance threshold of $p < 0.05$. Complete hierarchical clustering of inhibition data was performed by SRplot [Tang et al., 2023].

8 Results

8.1 Toxoplasmosis

8.1.1 Review of the literature

At first we have reviewed the literature for selecting the most characteristic ultrasound marker(s). Altogether 1480 hits were received. 718 duplicates and 38 hits were excluded from processing due to foreign language. 114 animal or in vitro studies were also excluded. 610 abstracts were reviewed, of which 505 were excluded based on the guidelines detailed above.

For 105 studies, the full text was analyzed. Of these, 11 studies were excluded because the full text was not available and in 83 cases the full text was not relevant to the research question. A summary table of the cases described in the processed studies was prepared (**Table 1 and 2**). The mean maternal age in the studied cases was 29.27 ± 6.03 years ($n=15$). 36 cases were diagnosed with positive maternal toxoplasma IgM, one case was diagnosed postnatally. There were 30 cases with positive toxoplasma PCR (from amniocentesis), 6 cases with no data, 1 case with no amniocentesis due to postnatal diagnosis. The mean time of seroconversion ranged from 11.42 ± 9.75 to 20.21 ± 7.47 weeks (predominantly in the second trimester). The first fetal ultrasound abnormality was discovered at 25.86 ± 8.54 weeks of gestation ($n=22$). The most frequent prenatal ultrasound signals suggestive of congenital toxoplasma infection based on the available literature were tabulated and the reports were summarized. Of the 37 cases summarized, 4 cases of hydrops fetalis, 3 cases of ascites, 2 cases of pericardial effusion, 2 cases of pleural effusion and 3 cases of hyperechogenic bowel were described. Cerebral parenchymal abnormalities were diagnosed in 15 cases, and ventriculomegaly in 19 cases. Hepatosplenomegaly was found in 3 cases. Among other abnormalities, single umbilical artery (SUA) and SGA were present. The results are summarized in **Table 3**. Ventriculomegaly cases were classified as mild, moderate and severe. Where an exact figure was not available, ventriculomegaly between 10 and 12 mm was considered mild, ventriculomegaly between 12 and 15 mm was considered moderate, and ventriculomegaly above 15 mm was considered severe. The mean was 12.69 ± 2.62 mm (mostly with moderate severity). The distribution is shown in **Table 4**. In 5 of 37 cases examined, there are no data on whether prenatal antibiotic treatment was given. In 4 of 32 cases where data were available, pregnant women did not receive prenatal treatment. In 28 cases where antibiotic treatment was given, sulfadiazine was used in 1 case, sulfadiazine and pyrimethamine were administered in 19 cases, and the exact combination of prenatal treatment was not indicated in 8 cases. There are data on control ultrasound examination in 8 cases. Here, the previously seen abnormalities were aggravated, although ascites decreased on control ultrasound examination and ventriculomegaly progressed in some cases. Ophthalmological abnormalities tended to be mild-to-moderate, whereas neurological abnormalities tended to be moderate-to-severe. The results are presented in **Table 5**. We investigated whether the severity of complications was related to any prenatal factors (ultrasound findings, treatment). Our results are shown in **Table 6**. As it can be seen, significant results were observed only for ventriculomegaly and cerebral parenchymal abnormalities, where there was a weak positive correlation between ventriculomegaly and outcome.

The test showed a weak negative correlation between brain volume abnormalities and outcome.

Table 1. Summary of the processed studies. Prenatal examinations and ultrasound abnormalities during pregnancy in case of recent Toxoplasma infection

Author						First ultrasound signs								
	Maternal age	Maternal IgM	Maternal PCR	Time of seroconversion	First US (gestational week)	Hydrops fetalis	Ascites	Pleural fluid	Pericardial fluid	Hypercholesterolemia	Brain parenchymal abnormalities	Ventriculomegaly	Hepato-splenomegaly	Other
Friedman et al 1999 [12]	26	ppp	p	N/A	27	x	x	x	x	x	n	10 mm	n	-
Pedreira et al 2002 [13]	16	ppp	p	N/A	20	n	n	n	n	n	n	n	n	-
Senat et al 1999 [14]	36	ppp	p	N/A	31	n	n	n	n	n	ipsilateral hyperechogenicity	16 mm unilateral	n	-
Hernandez-Andrada et al 2002 [15]	N/A	ppp	p	N/A	22	n	n	n	n	n	n	mild	n	-
	N/A	ppp	p	N/A	30	n	n	n	n	n	n	severe	n	-
	N/A	ppp	p	N/A	24	n	n	n	n	n	n	severe	n	-
	N/A	ppp	p	N/A	28	x	x	x	x	n	n	n	n	-
	N/A	ppp	p	N/A	31	n	n	n	n	x	n	mild	n	-
Malingier et al 2011 [16]	32	ppp	p	N/A	N/A	x	n	n	n	n	n	severe	n	-
	33	ppp	p	N/A	N/A	n	n	n	n	n	n	severe	n	-
	31	ppp	p	N/A	N/A	n	n	n	n	n	n	n	n	-
	24	ppp	p	N/A	N/A	n	n	n	n	n	n	mild	n	-
	29	ppp	p	N/A	N/A	n	n	n	n	n	n	severe	n	-
	33	ppp	p	N/A	N/A	n	n	n	n	n	n	mild	n	-
	34	ppp	N/A	28-30	N/A	n	n	n	n	n	n	severe	n	-
	26	ppp	N/A	6-20	N/A	n	n	n	n	n	n	mild	n	-
O'Connor et al 2012 [17]	36	ppp	N/A	1-8	12	n	n	n	n	n	n	n	n	-
Blaakaer et al 1986 [18]	28	PN	PN	PN	33	n	x	n	n	x	n	n	n	-
Estrada 2017 [19]	19	ppp	N/A	1-6	11	n	n	n	n	n	n	n	n	-
Desai et al 1994 [20]	35	p	N/A	12. hét	30	n	n	n	n	n	n	14 mm	n	AUS

Table 1. Summary of the processed studies. Prenatal examinations and ultrasound abnormalities during pregnancy in case of recent *Toxoplasma* infection (continued)

Author						First ultrasound signs								
	Maternal age	Maternal IgM	Maternal PCR	Time of seroconversion	First US (gestational week)	Hydrops fetalis	Ascites	Pleural fluid	Pericardial fluid	Hypercholesterolemia	Brain parenchymal abnormalities	Ventriculomegaly	Hepatosplenomegaly	Other
Dhombres et al 2016 [21]	N/A	p	p	22-27	33	n	n	n	n	n	5-10 parenchymal nodular foci; 1 thalamic nodular focus;	n	x	-
	N/A	p	p	22-27	34	n	n	n	n	n	7 parenchymal nodular foci; bilateral hyper-echogenicity of the germinal matrix	n	x	-
	N/A	p	p	10-18	23	n	n	n	n	n	3 parenchymal nodular foci;	n	n	-
	N/A	p	p	11-20	29	n	n	n	n	n	2 parenchymal nodular foci;	n	n	-
	N/A	p	p	20-24	31	n	n	n	n	n	<5 parenchymal nodular foci;	n	n	-
	N/A	p	p	14-20	31	n	n	n	n	n	1 parenchymal focus;	n	n	-
	N/A	p	p	19	31	n	n	n	n	n	10 parenchymal nodular foci; extensive echogenic areas of the white matter in subcortical, periaqueductal and periventricular regions;	n	x	-
	N/A	p	p	1-22	26	n	n	n	n	n	10 parenchymal nodular foci;	n	n	-
DiCarlo et al 2011 [22]	N/A	p	N/A	25	N/A	n	n	n	n	n	calcification	n	n	-
	N/A	p	p	23	N/A	n	n	n	n	n	calcification	n	n	SGA
	N/A	p	p	26	N/A	n	n	n	n	n	calcification	n	n	-
	N/A	p	p	20	N/A	n	n	n	n	n	calcification	x	n	SGA
	N/A	p	p	10	N/A	n	n	n	n	n	n	x	n	SGA
	N/A	p	p	26	N/A	n	n	n	n	n	n	x	n	SGA
	N/A	p	p	22	N/A	n	n	n	n	n	calcification	x	n	SGA
	N/A	p	p	12	N/A	n	n	n	n	n	n	x	n	SGA

Table 2. Summary of the cases of the studies processed. Control ultrasound examinations and postnatal follow-up in cases of recent gestational toxoplasmosis (continued)

Author	Therapy	Control US during pregnancy				TOP / intrauterine exitus	Follow-up		
		Time of the follow-up US (GW)	Follow up US	Time of the 2 nd follow-up US (GW)	2 nd follow-up US		Ophthalmic complication	Postnatal imaging	Follow-up
Malinger et al 2011 [16]	received	-	-	-	-	in utero 35. héten	-	-	-
	received	-	-	-	-		choroidoretinitis	CT: hydrocephalus, intracranial calcification	development delay, epilepsy, blindness
	received	-	-	-	-		choroidoretinitis	US: hydrocephalus, intracranial calcification	-
	received	-	-	-	-		choroidoretinitis	CT: hydrocephalus, intracranial calcification	development delay, epilepsy, blindness
	received	-	-	-	-		choroidoretinitis	CT: hydrocephalus, intracranial calcification	development delay, epilepsy, blindness
	received	-	-	-	-		choroidoretinitis	CT: intracranial calcification	-
	received	-	-	-	-		choroidoretinitis	US and MRI: hydrocephalus, intracranial calcification	development delay
	received	-	-	-	-		-	-	postnatal exitus in 48h
O'Connor et al 2012 [17]	not received	28	ventriculomegaly	30	progressive ventriculomegaly		-	MRI: complete destruction of the posterior, parietal and occipital lobe, decreased white matter, subcortical cysts, intracranial calcification Abdominal US: hepatosplenomegaly	N/A

Table 2. Summary of the cases of the studies processed. Control ultrasound examinations and postnatal follow-up in cases of recent gestational toxoplasmosis (continued)

Author	Therapy	Control US during pregnancy				TOP / Intrauterine exodus	Follow-up		
		Time of the follow-up US (GW)	Follow up US	Time of the 2 nd follow-up US (GW)	2 nd follow-up US		Ophthalmic complication	Postnatal imaging	Follow-up
Blaakaer et al 1986 [18]	not received	-	-	-	-		microphthalmia (left sided) and periferial chorioretinitis	US: mild hydrocephalus	
Estrada 2017 [19]	not received	3. trimester	unilateralis szájpadhasadék	-	-		ipsilateral anophthalmia and 3 rd type craniofacial cleft palate		normal neurodevelopment
Desai et al 1994 [20]	P + S	33	progressive ventriculomegaly	34	ventriculomegaly and polyhydramnios		bilateralis chorioretinitis	US: lateral ventriculomegaly, intracranial calcificatio	
Dhombres et al 2016 [21]	P + S	-	-	-	-		normal	normal	normal
	P + S	-	-	-	-		ventriculomegaly and polyhydramnios		normal neurodevelopment
	P + S	-	-	-	-		right sided chorioretinitis, right sided ambyopia		normal neurodevelopment
	P + S	-	-	-	-		normal		normal
	P + S	-	-	-	-		right sided chorioretinitis		normal neurodevelopment
	P + S	-	-	-	-		normal		normal
	P + S	31	progressive hepatosplenomegaly	-	-	33 rd week	-	-	hepatosplenomegaly, pulmonal laesion
	P + S	-	-	-	-	31 st week	-	-	no extracerebral malformation
	P + S	32	severe hydrops	-	-	34 th week	-	-	multi-visceral foetopathy

Table 2. Summary of the cases of the studies processed. Control ultrasound examinations and postnatal follow-up in cases of recent gestational toxoplasmosis (continued)

Author	Therapy	Control US during pregnancy				TOP / Intrauterine exitus	Follow-up		
		Time of the follow-up US (GW)	Follow up US	Time of the 2 nd follow-up US (GW)	2 nd follow-up US		Ophthalmic complication	Postnatal imaging	Follow-up
DiCarlo et al 2011 [22]	P + S	-	-	-	-	-		periventricular calcification	
	P + S	-	-	-	-	-		periventricular calcification	
	P + S	-	-	-	-	-		periventricular calcification	
	P + S	-	-	-	-	-		periventricular calcification	normal neuro-development
	P + S	-	-	-	-	-	bilateral chorioretinitis	CT: intraparenchyma laesion	
	P + S	-	-	-	-	-	bilateral chorioretinitis	CT: intraparenchyma laesion	
	P + S	-	-	-	-	-	unilateral chorioretinitis		
	P + S	-	-	-	-	-	bilateral chorioretinitis	CT: intraparenchyma laesion	

P = pyrimethamine S = sulfadiazine N/A = not available TOP = termination of pregnancy GW = gestational week

Table 3. Percentage distribution of prenatal ultrasound abnormalities detected

n = 37	positive	data not available	percentage
hydrops	4	1	11,1%
ascites	3	0	8,1%
pleural fluid	2	0	5,4%
pericardial fluid	2	0	5,4%
hyperechogenic bowels	3	0	8,1%
brain parenchymal abnormalities	15	0	40,5%
ventriculomegaly	19	0	51,35%
hepatosplenomegaly	3	0	15,7%

Table 4. Severity and incidence of ventriculomegaly during prenatal ultrasound examination

	Frequency (n)	Percentage
negative	18	48,65%
mild	6	16,21%
intermediate	1	2,70%
severe	7	18,92%
no data on its size	5	13,52%
Total	37	100,0%

Table 5. Distribution of ophthalmic and neurological complications during neonatological follow-up. The percentage was calculated for the follow-up results (n = 20)

Ophthalmic complications	Frequency (n)	Percentage
mild	11	55%
intermediate	6	30%
severe	1	5%
Neurological complications	Frequency (n)	Percentage
mild	2	10%
intermediate	3	15%
severe	3	15%

Table 6. Statistical analysis of the relationship between neonatal complications and prenatal ultrasound abnormalities and treatment using Spearman's coefficient on our data

	Spearman koefficiens	p
Hydrops	0.16	0.50
Ascites	0.03	0.903
Pericardial fluid	0.16	0.50
Pulmonal fluid	0.16	0.50
Hyperechogenic bowels	0.03	0.903
Brain parenchymal abnormalities	-0.69	0.001
Ventriculomegaly	0.61	0.001
Hepatosplenomegaly	-0.34	0.133
Prenatal therapy	-0.16	0.501

8.1.2 Prenatal investigations

At the Department of Obstetrics and Gynecology, Semmelweis University, Hungary, between 1996 and 2020, 238 cases of Toxoplasma infection were studied, where Toxoplasma infection was confirmed by serological testing, and Toxoplasma PCR was performed in amniotic fluid. The mean age of the pregnant women examined was 29.47 ± 5 years. In terms of the outcome of pregnancies, there were 219 deliveries and seven terminations of pregnancy (TOP). In 230 of the 238 cases, Toxoplasma DNA was not detected by PCR in the amniotic fluid. TOP was chosen in only two of these cases (0.8%). We were able to detect Toxoplasma DNA in the amniotic fluid in a total of eight cases, and TOP occurred in five of these cases (62%). In 12 cases, no data were available on the outcome of pregnancies, with regard to pregnant women receiving further care at another institution (**Fig. 14**). A total of 25 cases of induced labour happened. The neonates were born at an average of 38.91 ± 1.94 weeks of gestation, and abortions occurred at an average of 22 ± 1.41 weeks of gestation. Of the 219 deliveries, 132 were spontaneous vaginal deliveries, there were 69 Caesarean sections and in 18 cases no information was available on the mode of delivery due to the healthcare provided at another institution. One case of intrauterine death was registered. The proportion of preterm births

was 8%. There were two IVF-ET, two ICSI pregnancies and five twin pregnancies during the study period.

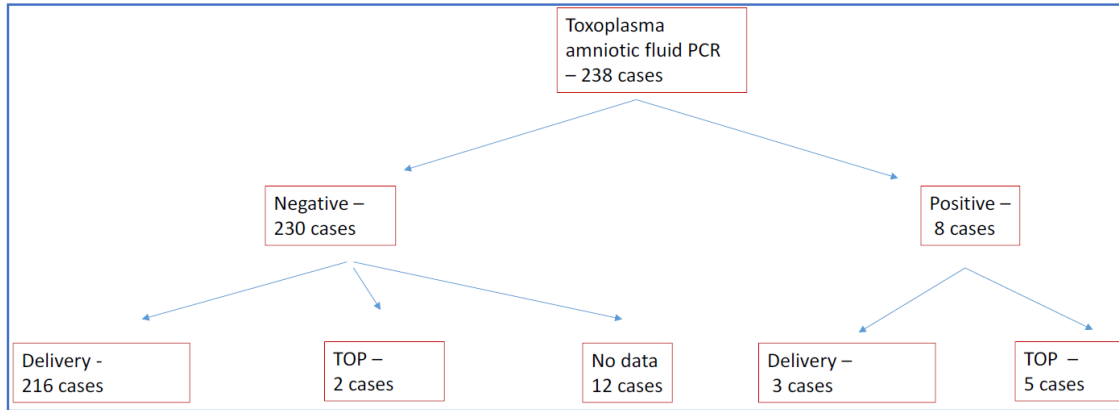


Figure 14. Patients participating in the study. TOP: termination of pregnancy.

8.1.3 Processing of Ultrasound Results

In total, 133 cases of ultrasound abnormalities were detected during pregnancy period, while 105 cases had no ascertained abnormalities during ultrasound examinations.

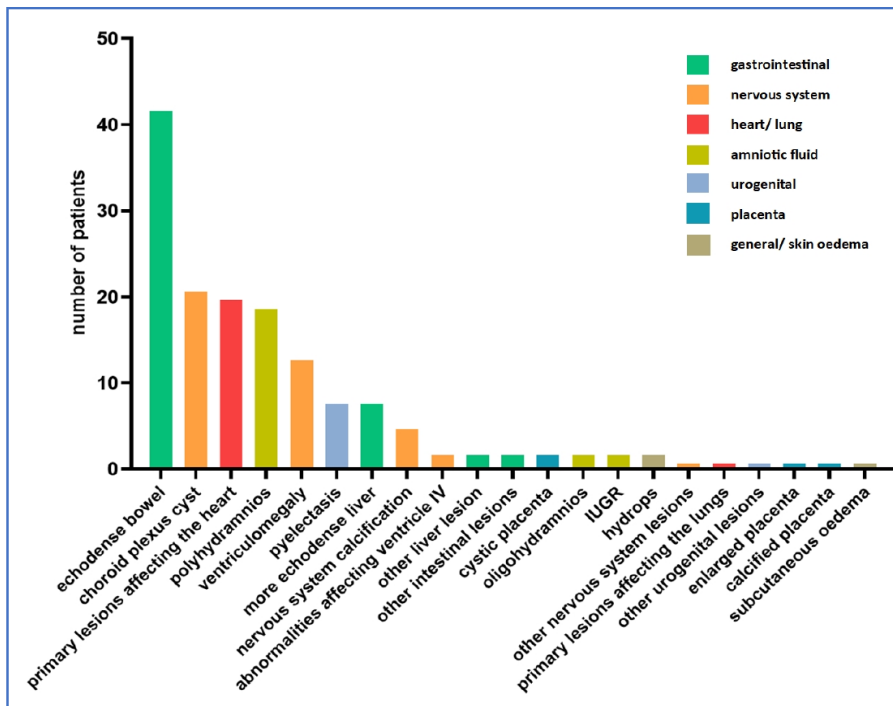


Figure 15. Distribution of ultrasound abnormalities (n = 105 cases).

Figure 15 shows the distribution of ultrasound abnormalities. Among amniotic fluid abnormalities, polyhydramnios was dominant, being present in 19 cases (14%). Oligohydramnios was detected in two cases (1%). Fetal growth retardation was also established in two cases (1%). The total number of data is projected to the 133 positive findings. Fetal edematous abnormalities included two cases of fetal hydrops (1.5%) and one case of subcutaneous edema (0.8%). No thicker nuchal fold was observed in any of the cases. The most commonly affected organ system was the craniospinal one. A total of 42 abnormalities affecting nervous system were featured in 38 fetuses (29%) (four cases with two nervous system anomalies simultaneously). The most common anomalies consisted of choroid plexus cyst (21 cases, 15.8%) and ventriculomegaly (13 cases, 9.8%). Nervous system calcification was present in five cases (3.8%). A lesion involving ventricle IV was described in two cases (1.5%). In the sample studied, ventricle III, the posterior fossa and the cisterna magna were not involved in any of the cases. We examined the lesions detected in the thoracic organs. In total, 21 cases of ultrasound abnormalities were detected (15.8%). Among the thoracic organs, the heart was most frequently involved such as thicker ventricular wall, dilated right heart, wider heart chambers, pericardial fluid (20 cases, 15%). One case of pulmonary abnormality could be characterized (0.8%). Abnormalities involving urogenital system could be diagnosed in nine cases (6.8%). The most common of the abnormalities affecting the urogenital system was pyelectasia, with eight cases (6%). Abnormalities of the gastrointestinal system were also usual (52 cases, 39.1%). Echogenicity enhancement may often indicate a possible Toxoplasma infection, with eight cases of hyperechogenic liver (6%) and 42 cases of hyperechogenic bowels (31.6%). Further liver lesions were detected in two cases, and further intestinal lesions were also identified in two cases (1.5% each). Abnormalities affecting the placenta were less frequent, with a total of four cases where abnormalities concerning the placenta (4.5%) were identified by ultrasonography. One case of thickened placenta (0.8%), two cases of cystic placental abruptions (1.5%) and one case of calcified placenta (0.8%) were explored. Two cases of other lesions not classified in any of the above tables were described (1.5%). Two cases of lesions involving more than one organ system were found. Both cases showed intracranial calcification and an echodense liver. In 67 cases, sonographic follow-up revealed the worsening of lesions.

8.1.4 Processing of PCR Results

Serological testing of maternal blood showed IgG positivity in 234 out of 238 cases with positive IgM, whereas four cases had borderline (equivocal) IgM. From amniotic fluid sampling, eight cases of *Toxoplasma* were detected by PCR, and negative results were obtained in 230 cases. Of the positive PCR samples, five pregnancies were aborted, and three pregnancies were carried to term. In all three of the latter cases, a mature baby was born. Although the positive amniocentesis PCR rate was significantly lower, it can be seen that when the amniocentesis sample was negative, a higher proportion of pregnant women chose to be carried to term (Tab. 7).

Table 7. Outcome of pregnancies with recent Toxoplasma infection as a function of PCR results (n= 238).

	Amniotic Fluid PCR Positive n = 8	Amniotic Fluid PCR Negative n = 230
Delivery	3 (38%)	216 (93.9%)
Abortion	5 (62%)	2 (0.8%)
Stillbirth		1 (0.5%)

8.1.5 Neonatological Follow-Up

Neonatological follow-up was performed in 139 cases and 117 cases had no abnormalities during the follow-up, whereas 22 cases had detectable complications probably related to *Toxoplasma* infection. **Figure 16** shows the distribution of neurological and other abnormalities detected during follow-up. In all 22 cases, PCR testing of *Toxoplasma* in the amniotic fluid was negative. Nine cases had neurological complications. In the follow-up cases studied, there were five cases with ophthalmological complications.

Other complications included: three cases of frequent ear infections, one child with no hearing in the left ear and four children who developed jaundice after birth but healed following the blue light treatment, with no permanent sequelae. No postnatal deaths occurred in any of the cases. A chi-square test was used to investigate whether there was a correlation between prenatal factors and subsequent complications. The results are presented in Table 8 below.

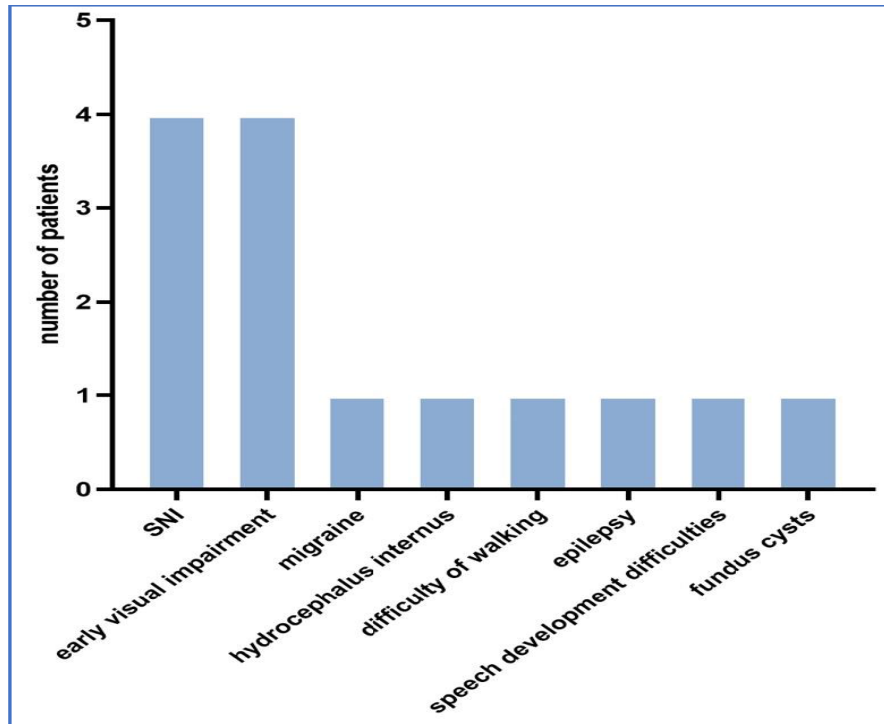


Figure 16. Distribution of neurological and other abnormalities detected during follow-up in Toxoplasmosis. SNI: Severe Neurological Injury.

Table 8. Associations between prenatal factors and subsequent complications

Prenatal Factors	Complications (n = 22)	No Complications (n = 117)	Total	<i>p</i>
Age over 37 years	1	13	14	0.407890
PCR positive	0	2	2	0.557217
Preterm birth	2	7	9	0.495630
Sex of the fetus (boy)	13	52	65	0.076257
Twin pregnancy	1	2	3	0.348785
IVF (ET or ICSI)	0	4	4	0.402803
Infection in the first trimester of pregnancy	4	13	17	0.257281

IVF: in vitro fertilization; ET: embryo transfer; ICSI: intracytoplasmic sperm injection.

As it can be seen from the above table, no significant correlation with the expected outcome could be demonstrated in any of the cases. However, the clinical relevance of this is questionable, as the rate of complications was low. We also investigated whether seropositivity associated with ultrasound abnormalities could predict the occurrence of neonatal neurological and ophthalmological complications. The results are illustrated in Tables 9 and 10. In cases with fetal subcutaneous oedema there was significant association with neonatal neurological complications.

Table 9. Association between neonatal neurological complications and fetal ultrasound malformations.

		Fetal Malformation on Ultrasound (218 Livebirths)				Total	Fisher's Exact Test
		Negative		Positive			
		Case	%	Case	%		
Neonatal neurological complications							
Subcutan oedema							
Negative	case	210	100.00%	0	0.00%	210	
	%	96.77%		0.00%			
Positive	case	7	87.50%	1	12.50%	8	
	%	3.23%		100%			
Total		217	99.54%	1	0.46%	218	0.037
Craniospinal malformations							
Negative	case	181	86.19%	29	13.81%	210	
	%	96.28%		96.67%			
Positive	case	7	87.50%	1	12.50%	8	
	%	3.72%		3.33%			
Total		188	86.24%	30	13.76%	218	0.697
Cardiovascular malformations							
Negative	case	192	91.43%	18	8.57%	210	
	%	96.00%		100%			
Positive	case	8	100.00%	0	0.00%	8	
	%	4.00%		0%			
Total		200	91.74%	18	8.26%	218	0.697
Pulmonary malformations							
Negative	case	209	99.52%	1	0.48%	210	
	%	96.31%		0.00%			
Positive	case	8	100.00%	0	0.00%	8	
	%	3.23%		0.00%			
Total		217	99.54%	1	0.46%	218	0.963
Urogenital malformations							
Negative	case	203	96.67%	7	3.33%	210	
	%	96.21%		100.00%			
Positive	case	8	100.00%	0	0.00%	8	
	%	3.79%		0.00%			
Total		211	96.79%	7	3.21%	218	0.767
Hepatic malformations							
Negative	case	203	96.67%	7	3.33%	210	
	%	96.21%		100.00%			
Positive	case	8	100.00%	0	0.00%	8	
	%	3.79%		0.00%			
Total		211	96.79%	7	3.21%	218	0.767
Gastrointestinal malformations							
Negative	case	170	80.95%	40	19.05%	210	
	%	95.51%		100.00%			
Positive	case	8	100.00%	0	0.00%	8	
	%	4.49%		0.00%			
Total		178	81.65%	40	18.35%	218	0.192

Table 10. Association between neonatal ophthalmological complications and fetal ultrasound malformations

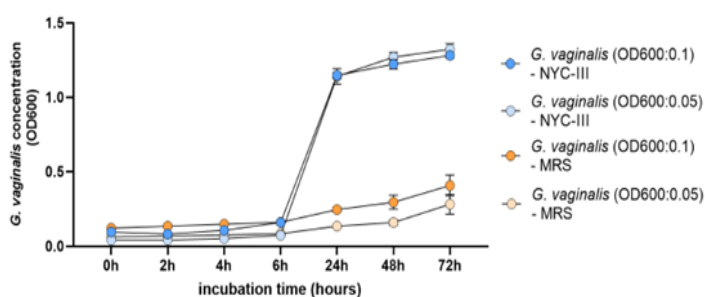
		Fetal Malformation on Ultrasound (218 Livebirths)				Total	Fisher's Exact Test
		Negative		Positive			
		Case	%	Case	%		
Neonatal ophthalmological complication							
Subcutan oedema							
Negative	case	209	99.05%	2	0.95%	211	
	%	96.76%		100.00%			
Positive	case	7	100.00%	0	0.00%	7	
	%	3.24%		0.00%			
Total		216	99.08%	2	0.92%	218	0.937
Craniospinal malformations							
Negative	case	181	85.78%	30	14.22%	211	
	%	96.28%		100.00%			
Positive	case	7	100.00%	0	0.00%	7	
	%	3.72%		0.00%			
Total		188	86.24%	30	13.76%	218	0.349
Cardiovascular malformations							
Negative	case	193	91.47%	18	8.53%	211	
	%	96.50%		100.00%			
Positive	case	7	100.00%	0	0.00%	7	
	%	3.50%		0.00%			
Total		200	91.74%	18	8.26%	218	0.542
Pulmonary malformations							
Negative	case	210	99.53%	1	0.47%	211	
	%	96.77%		100.00%			
Positive	case	7	100.00%	0	0.00%	7	
	%	3.23%		0.00%			
Total		217	99.54%	1	0.46%	218	0.968
Urogenital malformations							
Negative	case	204	96.68%	7	3.32%	211	
	%	96.68%		100.00%			
Positive	case	7	100.00%	0	0.00%	7	
	%	3.32%		0.00%			
Total		211	96.79%	7	3.21%	218	0.793
Hepatic malformations							
Negative	case	205	97.16%	6	2.84%	211	
	%	97.16%		85.71%			
Positive	case	6	85.71%	1	14.29%	7	
	%	2.84%		14.29%			
Total		211	96.79%	7	3.21%	218	0.207
Gastrointestinal malformations							
Negative	case	172	81.52%	39	18.48%	211	
	%	96.63%		97.50%			
Positive	case	6	85.71%	1	14.29%	7	
	%	3.37%		2.50%			
Total		178	81.65%	40	18.35%	218	0.622

8.2 Gardnerella infection

8.2.1 Growth kinetics of *G. vaginalis* and *Lactobacillus* spp. in MRS and NYC-III media

To compare the ability of MRS and NYC-III media to support *G. vaginalis* and *Lactobacillus* growth, we cultured both bacteria in parallel in each medium (**Fig. 17A**). *G. vaginalis* growth was monitored using two initial concentrations, 0.1 OD₆₀₀ and 0.05 OD₆₀₀. *G. vaginalis* growth was limited after 6 h culture in both media, indicating that the bacterium was in the lag phase. However, there was a dramatic difference at the 24 h time point. *G. vaginalis* reached ~1.14 OD₆₀₀ in NYC-III, while only minimal growth could be detected in MRS medium.

A



B

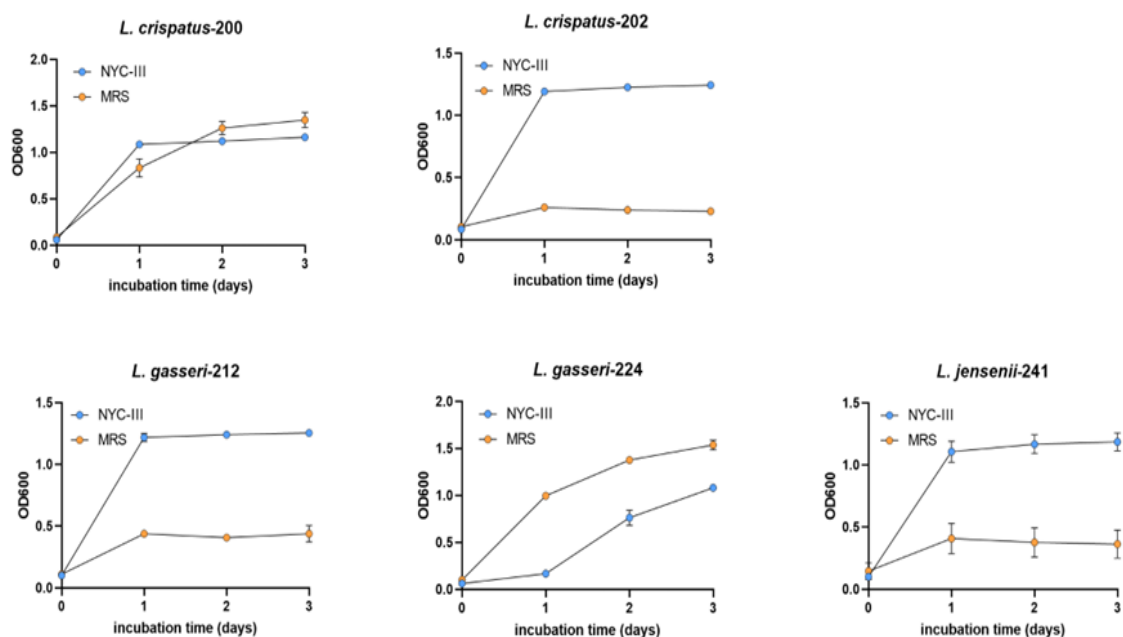


Figure 17. Growth kinetics of *G. vaginalis* and *Lactobacillus* spp. in MRS and NYC-III media. (A) *G. vaginalis* growth in MRS and NYC-III media. *G. vaginalis* was propagated in MRS or NYC-III media for 72 h at 37°C with 5% CO₂. (B) *Lactobacillus* spp. growth in MRS and NYC-III media. *Lactobacillus* strains were propagated in MRS or NYC-III media for 72 h at 37°C with 5% CO₂. OD₆₀₀ was measured at each time point (n=4). Data are presented as mean ± SD.

After 72 h incubation in NYC-III medium, *G. vaginalis* concentrations increased to approximately 1.3 OD₆₀₀, regardless of the initial inoculum size, whereas MRS medium only supported minimal growth of *G. vaginalis*, reaching 0.283 to 0.408 OD₆₀₀, depending on the initial inoculum size.

These data indicate that MRS medium may not support the growth of a low amount of *G. vaginalis*. *Lactobacillus* growth was also tested in the two media (**Fig. 17B**). Interestingly, only *L. gasseri*-224 grew better in the well-established *Lactobacillus* medium MRS than in NYC-III. *L. crispatus*-200 exhibited similar growth kinetics in both media, and the remaining three isolates grew better in NYC-III than in MRS. Altogether, these experiments showed that NYC-III supports the growth of both *G. vaginalis* and the *Lactobacillus* strains and is suitable for coculture experiments.

8.2.2 Development of a *G. vaginalis*-specific direct qPCR

G. vaginalis-specific primer pairs were evaluated for their performance in a direct qPCR. NYC-III medium containing *G. vaginalis* (0.1 OD₆₀₀) served as the template in the direct qPCR. HOT FIREPol EvaGreen qPCR Supermix was utilized in the qPCR, as this master mix had performed well in direct qPCR assays. The tested primer pairs exhibited significant differences in the sensitivity of *G. vaginalis* detection (**Fig. 18A**).

Primer-1 and Primer-4 produced the highest Ct values, and neither of these primer pairs could detect the bacterium above the annealing temperature of 65°C. Primer-2 demonstrated considerably lower Ct values compared to Primer-1 and Primer-4. qPCR with primer-3 resulted in the lowest Ct values, and, interestingly, the Ct values did not change significantly at higher annealing temperatures. Overall, Primer-3 produced the lowest Ct values, even at high annealing temperatures, and was selected for further qPCR using a 68°C annealing temperature. Specificity of Primer-3 became evident while using NYC-III medium containing *G. vaginalis* or one of the five *Lactobacillus* strains (**Fig. 18B**). Primer-3 also displayed a 4,096-fold dynamic range, using serial four-fold dilutions of *G. vaginalis* culture as direct template (**Fig. 18C**). When Ct values were plotted against the dilution factor, the linear regression slope was 2.083, indicating an average 4.3-fold concentration difference between the four-fold template dilutions. qPCR with purified DNA from *G. vaginalis* cultures demonstrated slightly higher sensitivity and a four-fold greater dynamic range than the direct qPCR.

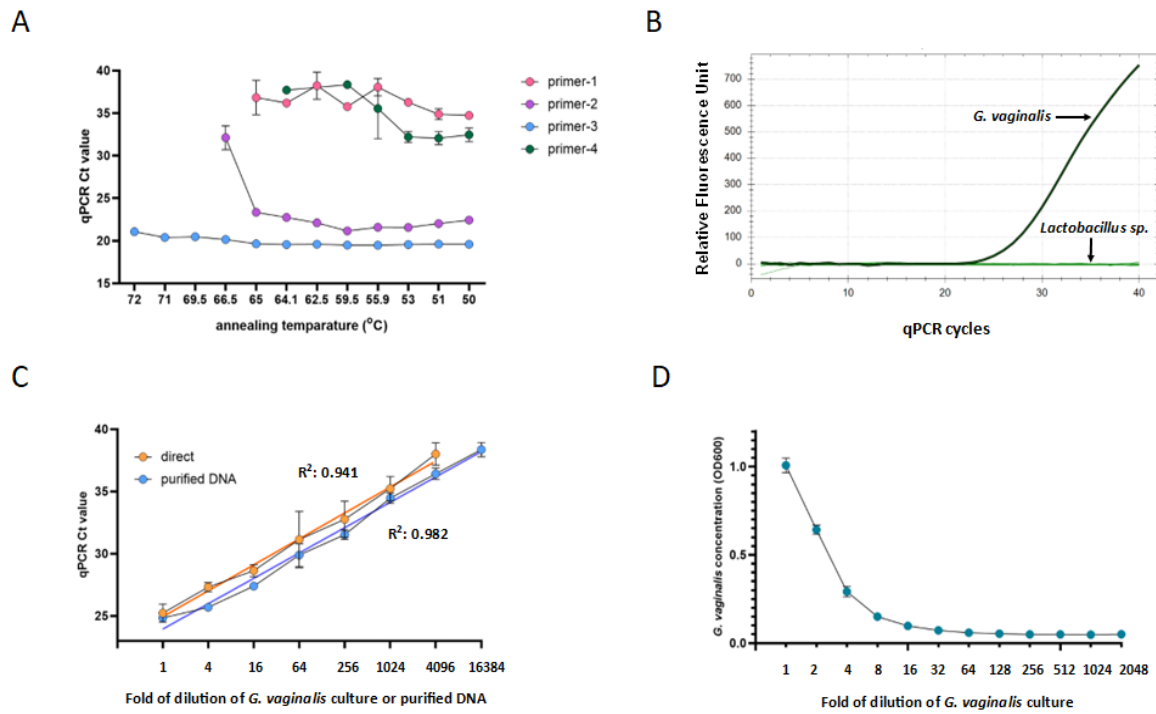


Figure 18. Development of a direct qPCR method to detect *G. vaginalis* in cocultures. **(A)** Impact of annealing temperature on the Ct values of *G. vaginalis* direct qPCR ($n=3$). **(B)** Specificity of *G. vaginalis* direct qPCR. Cultures of *G. vaginalis* and *Lactobacillus* isolates were used as templates in a *G. vaginalis*-specific direct qPCR setting. Representative amplification is shown. **(C)** Dynamic range of *G. vaginalis*-specific direct qPCR. Serial four-fold dilutions of *G. vaginalis* cultures and purified *G. vaginalis* DNA were used as templates in a qPCR setting ($n=3$). Linear regression was calculated for both templates, with R^2 values shown. **(D)** Dynamic range of spectrophotometry of *G. vaginalis* concentration. *G. vaginalis* was propagated in NYC-III medium, and the concentration was adjusted to 1.0 OD_{600} . Serial two-fold dilutions of the culture were performed, and the OD_{600} was measured ($n=3$).

These data indicate that the performance of the direct qPCR was comparable to that of the regular qPCR with purified DNA template and omitting the DNA purification step is feasible and may significantly simplify growth measurements. Additionally, a serial two-fold dilution series of *G. vaginalis* culture revealed that spectrophotometry had a dramatically lower dynamic range, showing an approximately 4-fold dynamic range (**Fig. 18D**).

8.2.3 Comparison of methods for testing *Lactobacillus*-mediated inhibition of *G. vaginalis*

First, we employed the well-established cell-free supernatant-mediated inhibitory assay to evaluate the inhibitory phenotypes of the *Lactobacillus* strains. Cell-free supernatants from each strain were tested at 50%, 25%, and 12.5% v/v (**Fig. 19A**).

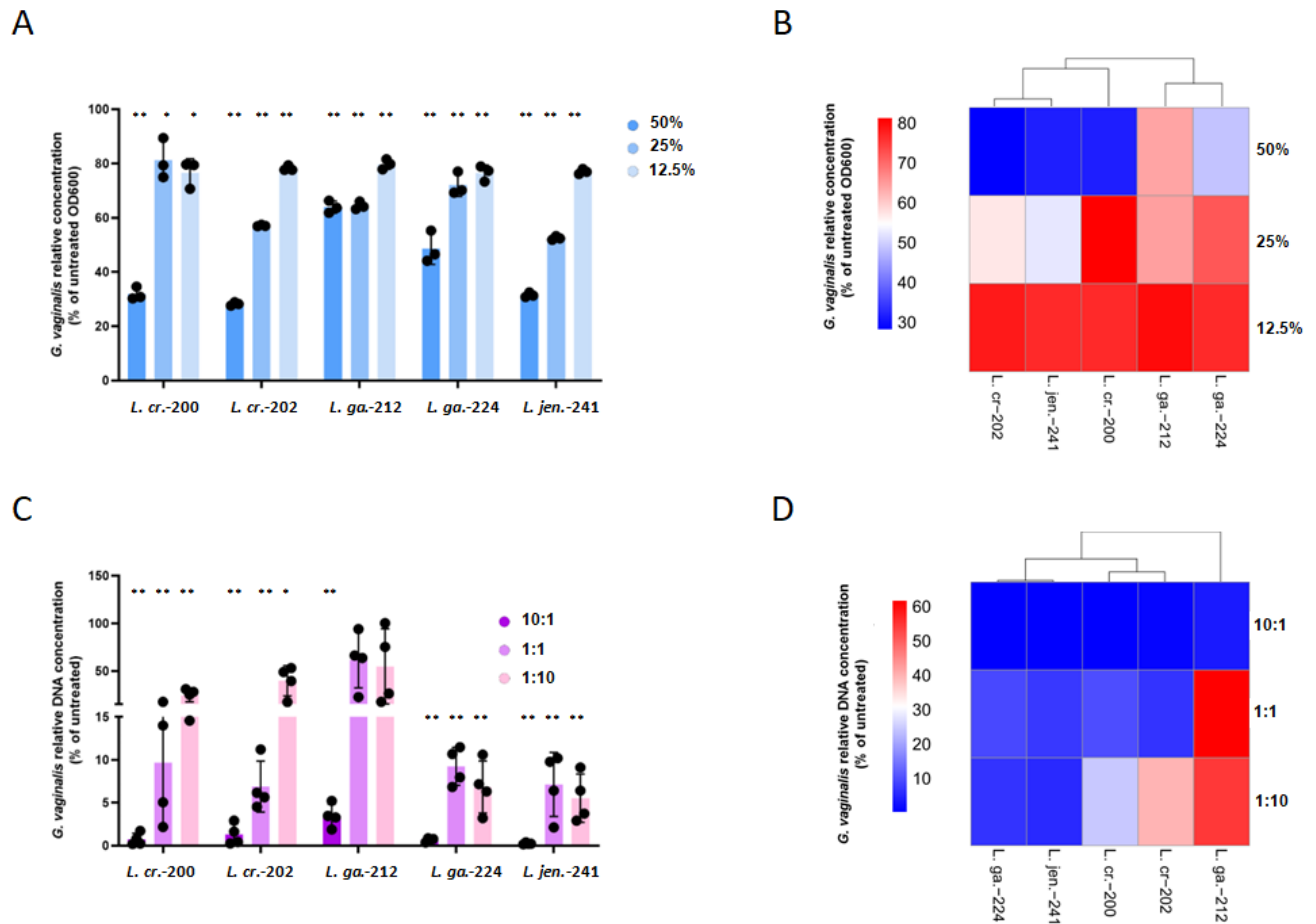


Figure 19. Comparison of methods for testing *Lactobacillus*-mediated inhibition of *G. vaginalis*. **(A)** Inhibition of *G. vaginalis* growth by *Lactobacillus* supernatants. *G. vaginalis* was cultured in NYC-III medium with 50%, 25%, and 12.5% v/v *Lactobacillus* supernatants for 48 h at 37°C with 5% CO₂. After 48 h of culture, the OD₆₀₀ of *G. vaginalis* cultures was measured and compared to the untreated control (n=3). Statistical comparisons of OD₆₀₀ values were performed using one-way ANOVA with a significance threshold of p<0.05. **(B)** Comparison of the inhibitory effect of *Lactobacillus* supernatants. This comparison was conducted through hierarchical clustering of the average *G. vaginalis* growth in the presence of *Lactobacillus* supernatants. **(C)** Inhibition of *G. vaginalis* growth in coculture with *Lactobacillus* spp. *G. vaginalis* and *Lactobacillus* strains were mixed in 10:1, 1:1 and 1:10 *Lactobacillus* sp./*G. vaginalis* ratios. Bacteria were cultured in NYC-III media for 48 h at 37°C with 5% CO₂. At the end of the coculture, *G. vaginalis*-specific direct qPCR was applied to detect *G. vaginalis* growth (n=4). Relative concentrations compared to *G. vaginalis* monocultures are shown. Statistical comparisons of qPCR Ct values between coculture and monoculture *G. vaginalis* were performed using a Student's t-test (p<0.05). **(D)** Comparison of the inhibitory effect of *Lactobacillus* strains. This comparison was conducted through hierarchical clustering of the average *G. vaginalis* growth in the coculture samples.

At 50%, three strains reduced *G. vaginalis* growth to approximately 28.2-31.9% of the control, while *L. gasseri* 224 and *L. gasseri*-212 were less effective, only reducing growth to 48.6-63.9%. At 25%, *L. jensenii*-241 and *L. crispatus*-202 exhibited the greatest inhibition,

limiting *G. vaginalis* growth to 52.5% and 57%, respectively. At 12.5%, all strains had a restricted growth to approximately 80% of the control (**Fig. 19B**).

For coculture experiments, we tested three initial *Lactobacillus*: *G. vaginalis* ratios, namely 10:1, 1:1, and 1:10. The direct qPCR method was employed to monitor *G. vaginalis* growth. *L. jensenii*-241 and *L. gasseri*-224 significantly inhibited *G. vaginalis* growth at all three inoculum ratios (**Fig. 19C**).

L. crispatus-200 and *L. crispatus*-202 showed marked growth inhibition only when there were either 10-fold more *Lactobacillus* or equal amounts of *Lactobacillus* in the coculture samples initially. *L. gasseri*-212 was the weakest inhibitor, exhibiting significant inhibition only when there was a 10-fold increase in *Lactobacillus* in the cocultures initially. *Lactobacillus* strains were then clustered based on their inhibitory activity in cocultures. *L. jensenii*-241 and *L. gasseri*-224 formed a high-inhibitor group, *L. crispatus*-200 and *L. crispatus*-202 formed a moderate-inhibitor group, and the weak-inhibitor *L. gasseri*-212 fell below (**Fig. 19D**).

9 Discussions

9.1 Toxoplasmosis

9.1.1 Ultrasound Examination

The diagnosical and treatment options for *Toxoplasma* infections have been addressed in a number of publications. A multicenter research group [Codaccioni et al., 2020] have evaluated the ultrasound findings of 88 pregnant women with confirmed *Toxoplasma* infection. In 45 (51.1%) cases, there was one or more craniocerebral abnormalities, in eight (9.1%) cases other organ systems were involved. The most common intracranial abnormalities were calcification (60 cases) and ventriculomegaly (44 cases), which worsened with advancing pregnancy.

Fetal ultrasound signs that may alert to intrauterine infection are nonspecific: intracranial calcification, ventriculomegaly, hydrocephalus, hepatosplenomegaly with or without calcification involving the gastrointestinal system, as well as hyperechogenic bowel. In addition, ascites, pericardial effusion, hydrops, and polyhydramnios may be present. Intrauterine growth restriction/small for gestational age (IUGR/SGA) is often observed [CMFN Barcelona Guideline 2024]. Although many of these infections produce similar fetal abnormalities and can be present with similar ultrasound abnormalities, the first line ultrasound screening seems to be a non-invasive, cost-effective, suitable method for screening a large number of pregnant women during short time. That is the reason why we intended to find an adequate US marker.

In our study, intracranial abnormalities were also very common (29%), although we have found lower amount of cases than published in the literature. Similarly, calcification and ventriculomegaly belonged to the most common intracranial abnormalities, whereas ventriculomegaly was more common in our study (ventriculomegaly: $n = 13$, calcification: $n = 5$). We did not investigate the progression of ventriculomegaly. The most common extracranial abnormalities were liver abnormalities (mainly hepatomegaly, 14 cases), followed by increased intestinal echogenicity (11 cases). In the period processed, the rate of increased intestinal echogenicity was much higher in our study (31.6% of cases) than in the report of Codaccioni et al. [Codaccioni et al., 2020]. Hohlfeld et al. have studied 89 pregnancies with confirmed *Toxoplasma* infection. The most common abnormality ($n=25$) was ventriculomegaly, followed by intracranial calcification ($n=6$). Their data are virtually identical with our data, but in percentage, these were lower in our study.

Ultrasound can detect the most severe abnormalities (except retinal abnormalities), but certain markers of impairment may not be visible until the later phase of pregnancy. In cases where

there is severe damage to the retina, it can also be associated with cerebral abnormalities. The following ultrasound findings are counted as the most characteristic: 1.) ventriculomegaly. It has generally a poor prognosis, especially if it is classified as a severe hydrocephalus (> 15 mm) 2.) Hyper-refringent intraparenchymal foci or nodules (cerebral calcifications). When these features appear isolated, the prognosis is uncertain. In general, they are not associated with neurological sequelae, but they increase the risk of chorioretinitis. 3.) Porencephaly (due to periventricular parenchymal destruction) 4.) microcephaly 5.) ascites 6) hydrops 7) hepatomegaly 8) splenomegaly 9) intrahepatic calcifications, and 10) thickened placenta. There is no evidence of association with fetal growth retardation. [CMFN Barcelona Guideline, 2024]

Placental abnormalities were also analyzed, in which 11 cases were specified as thickening and two cases as increased calcification. In our study, there was only one of each of these cases [Hohlfeld et al., 1991]. The reason for the differences in the results may be in consequence of the fact that *Toxoplasma* infection causes a wide variety of developmental abnormalities, thus leading to a huge spectrum of ultrasound findings. We may conclude that although the percentage of variation in each organ system is perceptible within a wide range, the results processed in our study are proportionally in line with the literature.

9.1.2 PCR Tests from Amniotic Fluid

In 2016, De Oliveira Azevedo conducted a comprehensive meta-analysis of studies, in which PCR testing was completed on amniotic fluid samples from pregnant patients to detect *Toxoplasma*. A total of 4171 cases from 20 studies were surveyed. Three studies investigated the gestational age at acute infection, ranging from 15.6 to 19.6 weeks, which is also consistent with our results (18.8 ± 2.3 weeks of gestation). The results reflect that the sensitivity of both IgM and PCR screening increases with advancing gestational age; nonetheless, there are no significant statistical results. All the reviewed literature points in this direction, and our study has also confirmed this finding. According to current professional recommendations, amniocentesis was only performed in the second trimester, so seroconversion rates could only be scanned then. However, based on the literature data, the sensitivity of amniocentesis in the first trimester is inherently much lower, because placental permeability is low and few fetal cells can be recovered from the amniotic fluid. Overall, both the meta-analysis and our study substantiate that the detection of *Toxoplasma gondii* DNA by PCR from amniotic fluid is currently the most reliable, rapid and low-invasive method concerning intrauterine *Toxoplasma* infection, even with the given limitations [de Oliveira et al., 2016].

9.1.3 Follow-Up Examination of the Newborn

Caceres et al. have investigated the neurological complications. While most infected newborns have developed no complications, among those with intracranial involvement, 8% to 12% of the cases appeared with intracerebral calcifications, 4% to 30% with hydrocephalus and 12% to 15% with chorioretinitis. Subsequent neurological involvement has been present in 12% of cases, proportionally consistent with our data [Caceres et al., 2024]. In a retrospective study, Reynolds et al. have investigated the presence and management of congenital macular lesion in their patients. In their practice, nine children required ophthalmic intervention, five of which had macular scarring. On average, the complaints occurred before the age of 3 years. All patients improved with ophthalmic intervention, regardless of the severity and size of the macular lesion. Their professional recommendation is that ophthalmic intervention for Toxoplasma infection with ocular complications can greatly improve the quality of life and is therefore recommended [Reynolds et al., 2020]. Ferreira et al. have published a literature review on the impact of congenital Toxoplasma infection on hearing [Ferreira et al., 2023]. This paper suggests that hearing impairment caused by Toxoplasma infection has both peripheral and central components. Research has also indicated an association between congenital Toxoplasma infection and mild to moderate hearing loss; however, these studies did not evaluate whether risk factors for hearing impairment other than congenital infection were present in the affected patients (e.g., prematurity, ototoxic medications, etc.). In our study, we found one case of hearing impairment and one case of recurrent ear infection during follow-up.

Regarding the fact that the review of the literature has not yet verified a link between recurrent otitis media and congenital Toxoplasma infection; it is still likely to be an independent factor [Ferreira et al., 2023]. There were 230 negative PCR results, but there were also 22 cases where complications still arose despite negative PCR findings. The study draws attention to the limitations of PCR tests. The PCR tests were performed at a specific time interval, during a certain period of pregnancy, and were not repeated at a later stage due to the risks of amniocentesis. Therefore, it could happen that the PCR result was negative at the time of the test, but later it could become positive, but this was no longer detected. In 12 cases, no information was available about the post-natal examinations. The reason for this was that these births took place in another institution and no data on the newborns were accessible after the birth. This small number of cases slightly limits the results compared to all cases.

9.2 *Gardnerella vaginalis*

The difference between coculture-based and supernatant-based inhibition tests was especially significant for *L. crispatus*-224, which showed poor inhibition in the supernatant-based assay but proved to be excellent inhibitor in the coculture assay. The opposite was true for *L. crispatus*-202, which exhibited good inhibition in the supernatant-based assay but was a less effective inhibitor in the coculture assay. These differences are not unexpected. *In vitro*, both measurement methods primarily assess secreted antimicrobial compounds, such as bacteriocins, H₂O₂, or D- and L-lactate [Chen et al., 2021; Boris et al., 2000]. On the other hand, *in vivo*, bacteria within the same microenvironment may influence each other's metabolism and antimicrobial activity. Production of antimicrobial compounds in the presence of competing bacteria offers an evolutionary advantage, but may incur a cost in the absence of competition. For example, the basal bacteriocin production of the vaginal isolate *L. gasseri* EV1461 was 0-160 bacteriocin unit/ml (BU/ml), while after coincubation with three different *Lactobacillus* spp. or *Propionibacterium avium*, the bacteriocin production reached 1280-2560 BU/ml. Similarly, bacteriocin production in *L. acidophilus* La-5 was stimulated by coculturing with viable, but not autoclaved, *Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus* [Tabasco et al., 2009]. *L. plantarum* J23 also showed inducible bacteriocin production against viable *Oenococcus oeni*, as well as various *Lactobacillus* and *Pediococcus* strains [Rojo-Bezares et al., 2007]. This context-dependent, quorum sensing-mediated production of antimicrobials, particularly bacteriocins, is well-documented among lactobacilli [Man et al., 2021; Chanos et al., 2016; Man et al., 2023], and may explain the observed differences of inhibitory activities detected by the two methods.

10 Conclusions

Screening for prenatal infections is important during pregnancy, because the earlier the exposure, the more serious the consequences are.

In toxoplasmosis the most common ultrasound abnormality affects the nervous system and the gastrointestinal system. In case of suspicion, in addition to indirect methods, it is recommended to carry out a PCR test, helping the pregnant woman to make a decision regarding pregnancy. We suggest universal screening at the time of 1st trimester blood test. In case of positive IgM, an alert should be generated to the clinician and the laboratory should automatically perform IgG avidity assay with the same sample. If the avidity is low or medium (de-

pending on the weeks), initiation of drug therapy is recommended until amniocentesis. If IgG is negative and IgM positive, it should be repeated after two weeks to differentiate between early infection and false positive IgM. In seronegative pregnant women, serology should not be repeated in the second or third trimester. In respect of the severity and appearance of complications, we did not find any prenatal factors with which we could prove a significant correlation. While ultrasound and PCR testing remain indispensable tools in managing toxoplasmosis during pregnancy, further research is needed to identify additional factors that may contribute to the variability in clinical outcomes. During follow-up, a multidisciplinary team that is experienced in pregnancies complicated by toxoplasmosis, must carry out the follow-up, care and subsequent management.

G. vaginalis screening is important during gestation. Our study suggests that cocultures which may reflect to *in vivo* microbial interactions should also be preferably used to evaluate *Lactobacillus*-mediated inhibition of *G. vaginalis* growth. Our direct qPCR method enables rapid and quantitative measurement of antimicrobial activity in cocultures. *Lactobacillus* therapy, an intimate wash that uses probiotics containing *Lactobacillus* strains, is generally considered safe during pregnancy. Our study suggests potential benefits, such as a reduced risk of overgrowth of *G. vaginalis* and thus bacterial vaginosis. This way it can improve the maternal health.

11 Limitations

11.1 Toxoplasmosis

A limitation of our study is that in the study period prenatal MRI was not widely used, and therefore in many cases ultrasound diagnosis was not confirmed. Moreover, the time of follow-up was variable in the reports.

11.2 *Gardnerella* infection

Our qPCR method was tested using the NYC-III medium. While the direct qPCR achieved good results with this medium, utilizing a more diverse range of media would demonstrate the robustness of the method more effectively. Another limitation is the number of *Lactobacilli* that we tested. Analyzing additional strains would clarify whether the difference in inhibitory activity mediated by cell-free supernatant versus coculture is common among various *Lactobacilli* species. In addition, more research is needed to determine optimal *Lactobacilli* strains and dosages.

12 New findings and results:

12.1 Toxoplasmosis

1. We recommend a universal toxoplasmosis screening at the time of maternal blood test in the 1st trimester.
2. The ultrasound screening is important in fetal life, but it is not considerably specific to diagnose fetal infections.
3. We did not find any specific prenatal ultrasound factors with which we could prove significant screening results for Toxoplasmosis.
4. Amniocentesis is an important diagnostic tool for fetal toxoplasmosis. It allows for the detection of *Toxoplasma gondii* DNA in the amniotic fluid using PCR, which can confirm or rule out fetal infection. This information is crucial for guiding treatment decisions for both the mother and the fetus.

12.2 Gardnerella infection

1. *Lactobacillus*-mediated inhibition of *G. vaginalis* growth was proven.
2. The direct qPCR was effective for measuring the antimicrobial activity.
3. We compared the inhibitory effects of *Lactobacillus* cell-free supernatants and those from *Lactobacillus-G. vaginalis* coculture on *G. vaginalis* growth. The cocultures that may reflect to *in vivo* microbial interactions better should also be used to evaluate *Lactobacillus*-mediated inhibition of *G. vaginalis* growth.
4. Lactobacillus therapy improves maternal health by reducing the risk of *G. vaginalis* overgrowth and bacterial vaginosis.

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15 Attachments-Publications

I.

II.

III.