UNIVERSITAT DE BARCELONA

Impact of Stress on Pregnancy Mental Health

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Impact of Stress on Pregnancy Mental Health

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List of abbreviations and acronyms

ASV: Amplicon Sequence Variant
BDNF: The neurotrophin brain-derived neurotrophic factor
BMI: Body Mass Index
COVID-19: Coronavirus 19 disease
HPA: Hypothalamic – Pituitary - Adrenal
MBSR: Mindfulness-Based Stress Reduction
PREDIMED: PREvención con DIeta MEDiterránea
PSQI: Pittsburg Sleep Quality Index
PSS: Perceived Stress Scale
RT–PCR: Reverse-Transcription Polymerase Chain Reaction
SARS-CoV-2: Severe acute respiratory coronavirus 2
SGA: Small-for-Gestational-Age
STAI: State-Trait Anxiety Inventory
WHO: World Health Organization
WHO-5: World Health Organization Well-being Index

1. List of articles

The present thesis has been structured following the normative for PhD thesis, as a *compendium of publications format,* to obtain the degree of Doctor in Medicine. The thesis consists of 5 objectives and 5 articles:

Article 1:

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Irene Casas*, Ayako Nakaki*, **Rosalia Pascal***, Sara Castro-Barquero, Lina Youssef, Mariona Genero, Leticia Benitez, Marta Larroya, Maria Laura Boutet, Giulia Casu, Alex Gomez-Gomez, Oscar J. Pozo, Ivette Morilla, Anabel Martínez-Àran, Eduard Vieta, María Dolores Gómez-Roig, Rosa Casas, Ramon Estruch, Eduard Gratacos, Fàtima Crispi, Francesca Crovetto. *These authors equally contributed. ***"Effects of a Mediterranean Diet Intervention on Maternal Stress, Well-Being, and Sleep Quality throughout Gestation – The IMPACT-BCN Trial."***

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Article 4:

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Article 5:

Selma-Royo M, Crispi F, Castro-Barquero S, Casas I, Larroya, M, Genero M, Paulés C, Benitez L, Youssef L, **Pascal R**, Dacal M, Nakaki A, Martín-Asuero A, Oller-Guzmán MT, Arranz A, Vieta E, Casas R, Estruch R, Gratacos E, Collado MC, Crovetto F. ***"Effects of Mediterranean diet or Mindfulness Based-Stress Reduction during Pregnancy on Maternal Gut and Vaginal Microbiota. A sub analysis of the IMPACT BCN trial".***

Status: Submitted, AJOG MFM

Impact factor: 7.1, Quartile: 1st

2. Summary

Títol: Impacte de l'Estrès en la Salut Mental de la Gestació

Introducció: Les alteracions de la salut mental perinatal i la qualitat del son són problemes habituals. A més, l'estrès pot induir *disbiosi* de la microbiota materna. L'evidència científica disponible posa de manifest l'impacte psicològic negatiu causat per l'esclat d'una pandèmia per una malaltia infecciosa i de les mesures de confinament i quarantena que se'n deriven, especialment per a les gestants. Les intervencions basades en canvis en l'estil de vida han estat postulades en els darrers anys com a possibles tractaments per a les malalties i problemes relacionats amb la salut mental. La microbiota materna té un efecte potencial en la protecció materna contra la malaltia i a la vegada modula l'eix intestí-cervell i, per tant, pot influir tant en el desenvolupament de determinades malalties com tenir un efecte negatiu futur en la funció cerebral i el comportament de la descendència.

Hipòtesi: La principal hipòtesi és que l'ambient a què es troba sotmesa la gestant pot tenir un impacte en la salut mental materna.

Objectius: El principal objectiu és el d'avaluar els efectes de diversos ambients materns durant l'embaràs sobre la salut mental materna.

Mètodes: S'utilitzen diverses cohorts per avaluar la salut mental materna durant l'embaràs i els efectes de l'ambient matern sobre ella a través de qüestionaris validats, informació clínica i biomarcadors: A) Una cohort de baix risc per descriure la salut mental materna en la

nostra població gestant. B) Un ambient matern negatiu: una cohort de gestants embarassades durant la pandèmia i confinament per COVID-19. C) Un ambient matern positiu: una intervenció basada en la dieta Mediterrània i un programa de reducció de l'estrès mitjançant *mindfulness* durant la gestació. De forma addicional, s'avalua també els canvis sobre la microbiota materna que s'hagin produït en aquestes circumstàncies.

Principals resultats: Es van observar alts nivells d'estrès en 23,1% de les participants al tercer trimestre, essent l'edat materna <40 anys (OR 2.02; 95% IC 1.08–3.81, p = 0.03), l'ètnia no-caucàsica (OR 2.09; 95% IC 1.19–4.02, p = 0.01) i no disposar d'estudis universitaris (OR 1.86; 95% IC 1.08–3.19, p = 0.02) els paràmetres més associats. Un total de 20,7% de dones tenien alts nivells d'ansietat al tercer trimestre, principalment influïts a l'anàlisi multivariat per l'antecedent de patologia psiquiàtrica (OR 3.62; 95% IC 1.34–9.78, p = 0.01) i no disposar d'estudis universitaris (OR 1.70; 95% CI 1.11–2.59, p = 0.01). En un 26,5% de les participants es va detectar malestar mental, influït de forma significativa per l'antecedent de patologia psiquiàtrica (OR 2.96; 95% IC 1.07–8.25, p = 0.04) i no disposar d'estudis universitaris (OR 1.74; 95% CI 1.10–2.74, p = 0.02). Finalment, es va detectar mala qualitat del son en el 81,1% i pitjor al final de l'embaràs respecte l'inici (p<0.001). Les gestants durant la pandèmia per COVID-19 tenien pitjors puntuacions al WHO-5 (median (IQR) de 56 (36–72) cohort de la pandèmia vs. 64 (52–76) cohort pre-pandèmia; p<0.001), amb un 42.8% de les dones presentant una puntuació negativa de benestar mental vs. 28% en la cohort pre-pandèmia (p<0.001). La presència d'una malaltia psiquiàtrica (OR 7.1; 95% CI 2.6–19, p<0.001), trobar-se al tercer trimestre d'embaràs (OR 1.7; 95% IC 1.5–2, p<0.001) o requerir ingrés hospitalari per COVID-19 (OR 4.7; 95% IC 1.4–16.7, p=0.014), van contribuir de forma significativa a l'anàlisi multivariat. Estar infectat pel virus SARS-CoV-2 no es va associar a pitjors puntuacions de benestar mental. En la intervenció amb dieta mediterrània

es va evidenciar, al final de la intervenció (34-36 setmanes) nivells significativament menors d'estrès i ansietat (PSS mean (SE) 15.9 (0.4) vs. 17.0 (0.4), p=0.035; STAI-anxiety mean (SE) 13.6 (0.4) vs. 15.8 (0.5), p=0.004) i millor qualitat del son (PSQI mean 7.0 $\pm$ 0.2 SE vs. 7.9 $\pm$ 0.2 SE, p=0.001) comparats amb el grup control. Les dones del grup intervenció amb dieta mediterrània tenien un augment significatiu en la ratio cortisona/cortisol en orina de 24h durant l'embaràs comparat amb el grup control (mean 1.7 $\pm$ SE 0.1 vs. 1.3 $\pm$ SE 0.1, p<0.001). La composició de la microbiota nasofaríngia en gestants amb infecció per SARS-CoV-2 (anticossos SARS-CoV-2 positius) va resultar diferent al comparar-la amb les gestants sense infecció (anticossos SARS-CoV-2 negatius)(p=0.001), amb una abundància relativa major de filaments *Tenericutes* i *Bacteroidetes* i una abundància major de la família *Prevotellaceae*. Les dones infectades presentaven un patró de microbiota diferent degut a la diversitat beta i una major riquesa alfa diversitat. Aquests canvis també es van observar en dones després d'una infecció aguda, amb RT-PCR negativa per SARS-CoV-2 però positiva per anticossos, suggerint una potencial associació entre SARS-CoV-2 i canvis persistents en la microbiota nasofaríngia. No es van trobar diferències significatives entre casos moderats i greus. Tant la intervenció amb un programa de dieta mediterrània com de reducció de l'estrès va augmentar la riquesa microbiana intestinal, i en el cas del programa de reducció d'estrès, també de la diversitat microbiana. Les dones en ambdós grups d'intervenció presentaven més abundància de gèneres relacionats amb la salut com el gènere *Blautia* i *Faecalibacterium* en el grup de dieta mediterrània i els gèneres *Lachnospiraceae* i *Ruminococcaceae* pel programa de reducció d'estrès. No es van trobar canvis en la microbiota vaginal.

Conclusions: L'estrès, l'ansietat, el malestar mental matern i les alteracions del son són freqüents i dinàmiques durant l'embaràs. L'ambient matern negatiu, com la pandèmia

COVID-19, empitjora la salut mental materna. L'ambient positiu, com una intervenció basada en la dieta mediterrània, millora l'estrès, l'ansietat, el benestar mental matern i la qualitat del son. La composició de la microbiota nasofaríngia materna va canviar en cas d'infecció per SARS-CoV-2. La intervenció materna amb un programa de dieta mediterrània o amb un programa de reducció de l'estrès mitjançant *mindfulness* produeixen canvis en la microbiota materna.

3. Introduction

Health is defined by the World Health Organization (WHO) as a "state of complete physical, mental and social well-being and not merely the absence of disease". Therefore, it's the sum up of physical health and mental health which contributes to an individual's overall wellness(1). In turn, the WHO defines mental health as the "state of mental well-being that enables people to cope with the stresses of life, realize their abilities, learn and work well, and contribute to their community"(1).

Mental health during pregnancy is, therefore, a crucial aspect of prenatal care. Pregnancy is expected as a time of emotional happiness and joy, however it can also be an emotionally challenging time for women, who could experience a range of negative affective states during this period with potential risks for their offspring. In this line, several studies have given evidence of the importance of good maternal health as a positive environment to fetal development.

3.1. Mental health on maternal status

Mental stress, anxiety, compromised well-being and sleep disturbances are fundamental and interconnected aspects of mental health. All of them can be found during pregnancy. Mental health, therefore, crucially depends upon affective states such as emotions, stress responses, impulses and moods(2), but also on personal life experiences and personality traits.

Mental ***stress*** can be medically understood as the "individual's perception of a stimulus as overwhelming" which derives in a response and a transformed state(3). It is a normal and natural response to stressors in life, but it can have adverse effects on a person's mental and physical health, especially when too intense or when prolonged in time.

Anxiety, in turn, is defined by the American Psychological Association as "an emotion characterized by feelings of tension, worried thoughts, and physical changes like increased blood pressure"(4). Both stress and anxiety are emotional responses. Although sometimes they are used as synonyms, stress and anxiety do not correspond exactly to the same concept, because stress is usually precipitated by an external factor, whereas anxiety is defined by the persistence of excessive worries even in the absence of the stressor(4).

Well-being, on the other hand, is considered "the quality and state of a person's life"(5) and consists of two different components: the feeling of health and feeling relatively robust and being able to carry out ones job and other tasks in a satisfactorily way(6). Therefore, mental well-being is a broader concept than stress and anxiety, which takes into account a person's overall mental health and emotional state going beyond the absence of mental illness. The concept of mental well-being is more dynamic and individualized and it also includes the ability to cope with stress, maintain positive relationships and enjoy life focusing on promoting positive mental health, resilience, and personal psychological thriving.

Finally, ***sleep quality*** is defined as an "individual's self-satisfaction with all aspects of the sleep experience"(7). It reflects how well-rested and refreshed a person feels after a night sleep, directly impacting on physical, mental and emotional functioning.

3.1.1. Prevalence of compromised maternal mental health

The reported prevalence for these antenatal mood conditions in the literature is variable, and actually the real prevalence of antenatal psychosocial stress and depression is still unclear(8,9). Around 20% of pregnant women could experience excessive worries for future events in pregnancy(6). Some studies report that up to 70% of pregnant women may refer symptoms of stress and anxiety during pregnancy. However, when applying diagnostic criteria for a major depressive disorder, this percentage could move between 10 and 16% (10,11). In a 2003 study, Rondó *et al.*, found high stress in between 22.1 and 24.6% of pregnant women in the three trimesters of pregnancy(12). In 2017, in a systematic review and meta-analysis of 102 studies involving 221.974 women, Dennis *et al.* found that the prevalence rate for self-reported anxiety symptoms in the first trimester was 18.2% and 24.6% in the third trimester. The overall prevalence of self-reported anxiety symptoms across the three trimesters of pregnancy was 22.9%. These percentages also decreased when employing diagnostic interviews: the prevalence rate for any anxiety disorder during the first trimester was 18% and 15% in the final two trimesters of gestation(13).

Such high percentages and the disparity found among studies have driven authors to speculate that symptoms of stress, anxiety and even depression can overlap some normal pregnancy feelings(11).

Finally, in a 2021 systematic review by Yin *et al.*, the overall pooled prevalence estimate for antenatal depression across 173 studies was 20.7% and 15% for major antenatal depression. The authors report that the prevalence of antenatal depression differed widely according to country income, assessment instruments and recruitment dates, finding higher prevalence in low- and middle-income countries, in self-report questionnaires when compared to

structured clinical interview and was also highest in studies conducted after 2010. However, no differences were found regarding the trimester of pregnancy(9).

There is no clear evidence of the prevalence of compromised well-being during pregnancy.

On the other hand, a highly variable prevalence of poor sleep quality in pregnant women has been also reported, ranging from 17% to 76%(14). This disparity could be due to dissimilar sample compositions and different methods and timings of assessments(15). Moreover, some authors have also postulated the possibility that the previous validated cut-off values for sleep questionnaires in general population may not be valid in pregnancies, thus requiring a higher score(15).

Such high prevalence percentage reported in the literature suggest the possibility of underassessment of these conditions by obstetric-care providers in daily clinical practice.

3.1.2. Mental health and sleep disturbances in maternal, pregnancy and neonatal outcome

The association of compromised maternal mental health and the consequences for maternal, pregnancy and offspring outcomes have been studied by numerous authors.

3.1.2.1. Maternal consequences of mental health during pregnancy

Pregnancy constitutes a unique time in terms of metabolic and physiological adaptations for the mother, with evidence that changes in the maternal brain constitute long-term

neuroanatomical modifications(16). Evidence has shown reductions in gray matter volume in regions subserving social cognition in healthy mother's brain suggesting an adaptative process to transitioning into motherhood. In the same study, follow-up of the participants showed that the changes observed in the maternal brain lasted for at least 2 years post-pregnancy(16). Interest is growing, in time, to assess if this neural plasticity that uniquely characterize the female brain during this period and the structural and functional changes that take place to produce behavioral adaptations to allow the mother to be responsible for the care of another life, also confer at the same time a vulnerability for the mother to develop mental disorders(17).

Women with antenatal depression are believed to be at higher risk for substance abuse(18) and higher depressive symptoms were also related to lower levels of healthy nutrition, higher levels of unhealthy nutrition(19), and postpartum depression. Postpartum depression is a prevalent mood disorder. The existence of antenatal mental health disorders, such as anxiety and other mood disorders, is a well-established psychosocial risk factors for postpartum depression(20–23). A systematic review of 28 studies reported that the prevalence of major depression disorder and minor depression ranged from 6.5–12.9% through the first 6 postpartum months, peaking at 2 and 6 months after delivery(21). Postpartum depression has also consequences for the mother with a lack of maternal bonding, and for the infant, as it can be related with impaired cognitive, emotional and behavioral development(24). When severe, perinatal depression could lead to suicide, which is the second-leading cause of maternal death in the postpartum period(24).

3.1.2.2. Mental health and pregnancy outcomes

Maternal mental stress, anxiety and compromised well-being during pregnancy have been associated with several adverse pregnancy outcomes including preterm birth(12,25–28), low birthweight(12,29–31), labor complications(32–34) or hypertension and preeclampsia(35).

Sleep disturbances such as poor sleep quality, shorter sleep duration and a later sleep midpoint during pregnancy have been associated to an increased risk of gestational diabetes(36,37).

A sleep duration of ≤6 hours has been associated to preterm birth(38). Moreover, in an observational study on more than 160 pregnancies, poor sleep quality assessed with the Pittsburg Sleep Quality Index (PSQI) questionnaire was found to be a predictor of preterm birth, with the largest effects in early pregnancy(14-16 weeks) and more modest effects in later pregnancy(30-32 weeks). With every one point increased on the PSQI, authors found that the odds of preterm birth increased by 25% in early pregnancy and 18% in later pregnancy(39). On top of that, in a prospective study of 688 patients, poor sleep quality during the third trimester was also associated to preterm birth(40). Du *et al.* found a relation between poor sleep during the first trimester and premature rupture of membranes(14).

In a prospective cohort study of 921 pregnancies, in 2021, Tang *et al.* described a progressively worsening of sleep quality as pregnancy advanced. Sleep duration was found to be negatively associated with both systolic and diastolic blood pressure and was also found to be associated with a higher uterine artery pulsatility index(41). Li *et al.*(40) found an association between poor sleep quality during first, second and third trimester of pregnancy and a higher risk of cesarean section. Similarly, in 2014 Hung *et al.* found a higher

risk of vacuum assisted delivery in patients with PSQI score >5 at third trimester of pregnancy(42).

Interestingly, Lee *et al.* in 2020 found that patients with PSQI score >5 had higher odds of stay in the neonatal intensive care unit and shorter birth length. However, in women <35 years, sleep quality was not associated with worst perinatal outcomes(43).

3.1.2.3. Maternal mental health and offspring outcomes

Maternal stress has been related to the activation of the Hypothalamic – Pituitary - Adrenal (HPA) axis causing a secretion of greater amount of glucocorticoids that enter fetal circulation and affect fetal HPA axis development and fetal glucocorticoid levels(44). Under this mechanism, maternal stress has been demonstrated to be a prenatal programming factor that adversely affects fetal neurodevelopment(45), compromising the socioemotional competence in early childhood, which provides a critical foundation for future academic skills and well-being(45,46). Mothers with antenatal depression and anxiety have 1.5-2 times greater risk of having children with behavioural difficulties(45). Moreover, maternal late pregnancy anxiety and stress has also been related to future children's general health: in a study on 174 mothers, Zijlmans *et al.* report that late pregnancy anxiety and cortisol was associated with children's respiratory and digestive illnesses until the age of 3.0–3.5 years(47).

The association of this maternal mental health conditions and sleep disturbances to such negative maternal, pregnancy and neonatal outcomes, stressed by the high prevalence of this conditions reported by literature, puts into evidence the importance of assessing maternal mental health in daily obstetric clinical practice.

3.2. Risk factors for negative maternal affective states

Although the firm recommendation to asses maternal mental health during pregnancy, the use of multiple questionnaires can be challenging in daily clinical practice, especially in high healthcare workload. Therefore, understanding the risk factors associated, may be critical in targeting those patients at higher risk and thus facilitating daily clinical practice as most of them can be identified at the beginning of pregnancy.

Different sociodemographic, medical and obstetrical risk factors for antenatal mood disorders have been postulated in previous published literature.

Sociodemographic variables such as age has been considered in multiple studies with inconsistent findings among them(48,49). Other sociodemographic variables considered were the maternal socioeconomic status and the educational level: Lancaster *et al.* in a 2010 systematic review, found a small association between low educational level and depression symptoms that could not be demonstrated in the multivariate analyses(50). Later, Biaggi *et al.* found low maternal educational level to be associated to anxiety and depressive symptoms(49).

As for ethnicity, socioeconomic status and employment, an unfavorable socioeconomic situation, unemployment and belonging to a minority ethnic group is associated to depression in several studies(9,48,49), but inconsistent results are described in others(49,50).

On the other hand, other factors such as smoking, alcohol intake and drug abuse showed inconsistent findings in their association to depression and sleep quality(9,14,49,50).

Lancaster *et al*. postulated socioeconomic factors like lack of social support, unintended pregnancy, availability of a private medical insurance, domestic violence, lower income and

lower education, single status, and poor relationship quality to be associated with a greater likelihood of antepartum depressive symptoms in bivariate analyses. However, only lack of social support and domestic violence demonstrated a significant association when performing the multivariate analyses(50).

Biaggi *et al.* found that socioeconomic factors such as lack of partner or social support, history of abuse or domestic violence and unwanted pregnancy were also predictors for antenatal depression and anxiety(49). Nasreen *et al.,* in a cohort study in 2018, postulated the perceived social and family support as a protective factor for antepartum depressive symptoms. On the other hand, they found intimate partner violence, poor relationship with husbands and husband's depression in current pregnancy to be risk factors for antepartum depressive symptoms(51). However, only a few quantitative reviews have been published with the aim to clarify the strength of these associations(9). In view of these gaps in the literature, Yin *et al.,* published a systematic review in 2021 with the objective to give a summary of association between potential factors and antenatal depression(9). They found lack of social support, single/separated/divorced status, experience of violence, unplanned pregnancy, unemployment and smoking to be associated with antenatal depression(9).

A personal medical history of anxiety, life stress and depression has strongly been associated to perinatal depression(9,48–51).

As for obstetric history, present/past pregnancy complications and pregnancy loss are factors associated with antenatal depression and anxiety(14,48,49) but also with inconsistent findings(50).

Such inconsistent findings among literature, suggest a complex multifactorial origin for these conditions etiology(50). Traylor *et al.* also suggest that although many women during pregnancy are exposed to acute and chronic stressors, not all of them have negative pregnancy outcomes, which could explain the variability found in pregnancy outcomes. It's the sum of an individual's prior positive and negative experiences and their reaction to these experiences that can conditionate whether a new distress can finally impact and disrupt normal homeostasis and result in an adverse outcome or have no final impact at all(52).

3.3. Self-reported validated questionnaires for assessment of maternal mental health

Because of all the data stated above, the diagnosis and screening of mental maternal health has been recommended by scientific societies for long time. For instance, the American College of Obstetricians and Gynecologists recommends to screen women at least once during the perinatal period for depression and anxiety symptoms using a standardized, validated tool(53). To do so, different assessment tools and scales can be used:

- The Perceived Stress Scale (PSS): The PSS is a brief scale which was designed to measure "the degree to which individuals appraise situations in their lives as stressful"(54). It consists of only 14 items which evaluate stress within the last 8 weeks. PSS scores are obtained by reversing responses to the four positively stated items (items 4, 5, 7, and 8) and then summing across all scale items. Because it is not a diagnostic instrument, there are no cut-offs for classification of the stress, but it gives a comparison instrument between people in the researchers sample(55). In our studies, the higher stress group in our cohorts was considered the 75th percentile at the first evaluation.

- The State-Trait Anxiety Inventory (STAI): The STAI questionnaire consists of two subscales: State Anxiety Scale (STAI-S) which evaluates the current state of anxiety, and the Trait Anxiety Scale (STAI-T) which evaluates individual aspects of "anxiety proneness". The STAI has 40 items, 20 items allocated to each of the S-State and T-Trait subscales. Range of scores for each subtest is 20-80, the higher indicating greater anxiety(56,57). The higher stress group is also usually considered to be the 75th percentile of the investigator cohort.

- The World Health Organization Well-being Index (WHO-5): The WHO-5 questionnaire consists of a five-item scale and it is used to rate quality of life and psychological well-being, according to the participant's feelings within the last 15 days. The raw score ranges from 0 to 25: where 0 represents worst possible and 25 represents best possible quality of life. Once the raw punctuation is obtained, it is then multiplied by 4 to obtain the final result. Women are then classified according to their well-being status as with a poor ($\leq$52) or favorable (>52) WHO-5 score(58). The existence of a cut-off score for the classification of subjects in "good" and "poor" mental well-being of this brief questionnaire, makes it an easier scale to use in daily clinical practice.

- The Pittsburgh Sleep Quality Index (PSQI): The PSQI assesses sleep quality and sleep disturbances over a month interval. The questionnaire contains 24 questions: 19 self-rated questions and 5 questions rated by the bed partner or roommate. However, only the self-rated questions are included in the scoring.

  The 19 self-rated items are combined to form seven component scores which are: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction. Each question has a range of 0-3 points (where 0 indicates no difficulty and 3 indicates severe difficulty). The seven component scores are then added up to obtain one global score (between 0-21 points): 0 indicating no

difficulty and 21 indicating severe difficulties. A global score greater than 5 defines a poor sleep quality in the questionnaire(59).

3.4. A negative maternal environment: COVID-19 pandemic and lockdown impact on maternal mental health

In December 2019, the first human cases of coronavirus 19 disease (COVID-19) were identified in Wuhan, China. The disease is caused by a severe acute respiratory coronavirus 2 (SARS-CoV-2), with the capacity to rapidly spread from human to human. On 30th January 2020, the WHO declared the COVID-19 outbreak a public health emergency of international concern and on 11th March 2020, a pandemic. As of 4th October 2023, 771.151.224 positive cases have been declared and 6.960.783 deaths across the world have been reported to WHO.

Strict public health measures directed to mitigate the spread of the disease, such as lockdown or transportation restrictions, were adopted in many countries in the world, especially during 2020. In May 2023 the WHO established COVID-19 was considered an ongoing health issue which no longer constituted a public health emergency of international concern and advised that it was time to transition to long-term management of the COVID-19 pandemic.

The COVID-19 has been broadly studied in pregnant women finding that pregnant women are mainly asymptomatic and the overall rate of pregnancy complications in women with SARS-CoV-2 infection has been found to be similar to non-infected women(60), with the exclusion of those in the third trimester closer to delivery, where the rate of complications is increased(60–62).

Previous evidence has revealed the negative psychological impact, in terms of anxiety, depression and post-traumatic stress associated to the outbreak of a pandemic and its consequences on the general population, particularly on people who have quarantined(63–66). Pregnant women are thought to be more vulnerable to this situation for several reasons: less prenatal visits, relatives not allowed in prenatal visits, uncertainty related to fetal transmission, social isolation…

During the COVID-19 pandemic, several studies were assessed on maternal mental health status, as the outbreak of the pandemic provided the opportunity to study the effects on pregnant population of a common external stressor. Such studies reported a compromised maternal mental health in pregnant population at the time(65,67–69). The overall reported prevalence in the literature for depressive and anxiety symptoms ranged around 15-19% and 11-31%, respectively(67,68).

Not many studies provided the comparison of the maternal mental health of pregnant women during the pandemic and previous cohorts of pregnant population before the pandemic. Mostly, these studies that did compare to previous cohorts, reported higher depression symptoms in women during the pandemic when compared to pre-pandemic cohorts(70–73).

Several risk factors of compromised maternal mental health were postulated: Wu *et al.* reported pre-pregnancy underweight, primiparous status, maternal age < 35 years old and middle income to be risk factors for depressive and anxiety symptoms assessed with the Edinburgh Postnatal Depression Scale. Surprisingly, full-time employment and having an appropriate living space were also described as risk factors in the same study(70). On the

other hand, Lebel *et al.* found that not getting necessary prenatal care, relationship strain and social isolation were associated to psychological distress, while better social support and physical activity were recognized as protective factors(74). In this line, Farewell *et al.* also described the uncertainty surrounding perinatal care and the lack of support network, as risk factors for depressive and anxiety symptoms. Partner support was also found to be a resilience source, as well as gratitude, virtual communications platforms, self-care behaviors, structures and routines, and being outdoors. The timing of pregnancy showed contradictory results among authors: Zeng *et al*. reported being in the third trimester of pregnancy at the time of the COVID-19 pandemic to be associated with a worse maternal well-being and that these women had even worse results than those compared to the post-partum period(67); Saccone *et al*., on the contrary, found worse results in anxiety and psychological tracts in pregnant women in the first trimester(69), whereas other authors did not find any differences according to gestational age(75–77). Symptoms of COVID-19 and its infection have been described as influencing factors for anxiety(78) and predictors for post-traumatic stress disorder(79). Therefore, the infection of SARS-CoV-2 could apparently increase the level of anxiety and worsen mental condition, but this hypothesis was not confirmed throughout the literature(77,80). Many authors reported a previous psychiatric disorder to be a risk factor for depression symptoms during the COVID-19 pandemic(71,81,82).

3.5. A positive maternal environment: the effect of lifestyle strategies during pregnancy on mental health

Current evidence states that implementing positive lifestyle strategies during pregnancy can have a significant effect on mental health(52). For all the reasons stated above, pregnancy

constitutes a unique situation in which the reduction of stress may have the potential for profound impact on the described maternal mental health and pregnancy outcomes(52).

However, only around 8% of patients with a diagnosed depression during pregnancy are believed to receive adequate treatment and data is limited regarding the remission rates of depression during pregnancy(52). Moreover, the use of pharmacological strategies during pregnancy could lead to negative outcomes in the offspring such as congenital heart disorders, neonatal persistent lung hypertension, neonatal sleep disorders, excessive crying(83), a more premature gestational age at birth and lower birthweight(84) among others, as there is evidence that these medications cross the placental barrier. On top of that, psychotherapy has also an important and well documented role in the treatment of mental health disorders; however, universal access to psychotherapy cannot be granted due to time, availability and economic reasons(52).

That is why, efforts in promoting lifestyle changes, which could lead to a reduction in maternal mental health issues during pregnancy have been addressed in recent years, in order to ensure the potential benefits of the stress reduction in pregnancy without assuming the potential risk of antidepressants use for the offspring and with the advantage of being affordable and widely available.

Some lifestyle choices that have been described as a potentially positive influence for pregnancy mental health include: regular exercise, a well-balanced and nutritious diet, adequate sleep, mindfulness-based stress reduction techniques, self-care, social support and prenatal education among others(52).

3.5.1. The effect of a Mediterranean Diet intervention program

The dietary intakes of pregnant women in developed countries was studied in a systematic review and meta-analysis of a total of 126,242 pregnant women(85). In this study, the authors concluded that energy and macronutrient did not match national recommendations: energy and fiber intakes were below recommendations and total and saturated fat were generally above the recommendations. Carbohydrate and polyunsaturated fat intakes were below to borderline low compared with recommendations. The authors finally stated that further research was required to assess the final implications of their findings, which would be unknown until studies comparing the maternal diet and the offspring health outcomes were conducted(85).

The Mediterranean diet is generally based on the daily intake of fruit and vegetables, whole grains, legumes, nuts, fish, white meats, and extra-virgin olive oil. It may also include moderate consumption of fermented dairy products, and a low intake of red meat(86).

The Mediterranean diet pyramid (figure 1) establishes daily, weekly and occasional dietary guidelines. It takes into account qualitative and quantitative elements for the selection of foods. The pyramid follows a pattern: at the base, foods that should sustain the diet (plant-based foods), and at the upper levels, foods to be eaten in moderate amounts (animal origin, rich in sugars and in fats)(87).

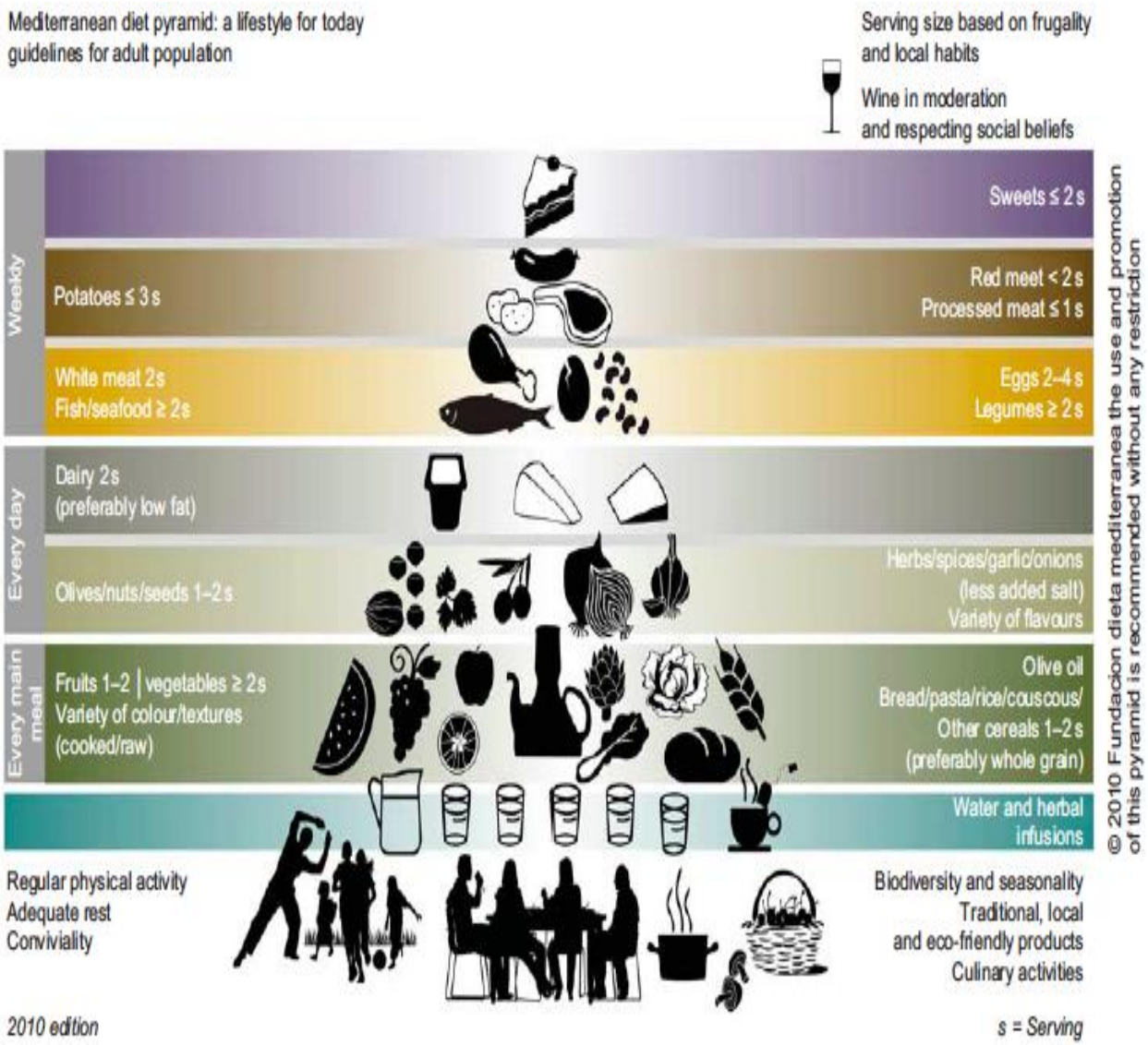

Figure 1: The Mediterranean Diet Pyramid. Authorship: ©2010 *Fundación dieta mediterránea*. The use and promotion of this pyramid is recommended without any restriction.

The three main daily meals should contain three basic elements, which can also be found throughout the day:

- Cereals. One or two servings per meal in the form of bread, pasta, rice, couscous and others. Preferably whole grain, since some valuable nutrients (magnesium, phosphorus, etc.) and fibre can be lost during processing.

- Vegetables. Present at lunch and dinner; or more than two servings per meal, at least one of the servings should be raw. A variety of colors and textures provide a diversity of antioxidants and protective compounds.

- Fruit. One or two servings per meal. Should be chosen as the most frequent dessert.

Also, a daily intake of 1.5–2.0litre of water is recommended. Dairy products are recommended in the form of low-fat yoghurt, cheese and fermented dairy products, although they could be an important source of saturated fat. Extra-virgin olive oil is a key element at the centre of the pyramid. It should be the principal source of dietary lipids and should be used for cooking as well as dressings (one tablespoon per person). Olives, nuts and seeds are good sources of healthy lipids, proteins, vitamins, minerals and fibre and should be reasonably consumed (a handful). Weekly, fish (two or more servings), white meat (two servings) and eggs (two to four servings) are good sources of animal protein. Consumption of red meat (less than two serving) and processed meats (less than one serving) should be in smaller quantity and frequency. The combination of legumes (more than three servings a week) and cereals are a healthy protein and lipid sources. Only on special occasions, sugary and unhealthy fats rich foods should be consumed(87).

The Mediterranean diet is well known for its positive effects on individuals health: randomized trials demonstrated its contribution to improved cardiovascular profiles and reduced major cardiovascular events in individuals at risk of diabetes, inflammatory based disorders, cancer or cognitive impairment(88–92).

Over the past decade, multiple research has focused on the role of the diet in the development and outcome of mental disorders(86). Understanding the relationship between diet and mood could be of vital importance in order to implement lifestyle changes as a treatment for both obesity and mental health, with numberless benefits for both mental and physical health.

A recent review based on 37 studies confirmed the association between (poly)phenols consumption and the risk of depression, and a reduction in the severity of depressive symptoms(93). Some authors hypothesized that a high-quality diet, rich in fiber, antioxidant dietary components and omega-3-polyunsaturated fatty acids, may be linked to a reduced risk of depression, anxiety, and stress(94), which could provide new potential methods for the treatment and prevention of mental disorders in general. Moreover, it has been described that a dysregulated redox signaling is a key factor in the pathophysiology of mental disorders, especially in depression, and increased reactive oxygen and nitrogen species were observed in these patients(95,96). In this line, both the individual antioxidant capacity and the production of reactive oxygen and nitrogen species is influenced by several dietary factors. Thus, a dietary intervention promoting plant-based foods that are rich in antioxidants, such fruits, vegetables, extra-virgin olive oil, and whole-grain cereals, may modulate the individual antioxidant capacity, explaining the improvements in mental well-being(95).

Several studies evaluated the effect of a Mediterranean diet intervention on the reduction in depressive symptoms and the improvement in quality of life in individuals with major depressive disorders: In 2017, Parletta *et al.,* tested a Mediterranean diet intervention supplemented with fish oil in 152 subjects with self-reported questionnaires of depressive

symptoms; they found that the Mediterranean diet group intervention had less symptoms of depression and improved mental health at 3 and 6 months of follow-up, with correlation to the Mediterranean diet-adherence of the subjects in the intervention group. Interestingly, they also found positive correlation with high levels of omega-3, low levels of omega-6 and mental health(97). Similarly, in a randomized controlled trial conducted by Jacka *et al.* in 2017, where they investigated a Mediterranean diet intervention as a possible treatment for major depression symptoms, authors report major improvement in the depression-reporting scales in intervention group compared to controlled group in a total of 67 individuals(98). In a secondary analysis of the *PREvención con DIeta MEDiterránea* (PREDIMED) study, a reduced risk in depression was observed in participants with type 2 diabetes allocated to the group receiving a Mediterranean diet supplemented by nuts(99).

During pregnancy, evidence has been provided regarding the potential beneficial effects that structured dietary interventions based on a Mediterranean diet can have, not only on pregnant women but also their offspring and the pregnancy itself(96,100). In a recent randomized clinical trial, pregnant individuals at high risk for small-for-gestational-age newborns who followed a structured Mediterranean diet intervention significantly reduced the incidence of newborns being born small (with birth weight below the 10^{th} percentile) and other perinatal complications(101).

However, there is still paucity of data regarding the potential benefits in maternal mental health of a dietary intervention during pregnancy. In 2022, Flor-Alemany *et al.* published a secondary analysis of the GESTAFIT trial, where they found that a greater Mediterranean diet adherence was negatively associated to negative affect and anxiety and positively associated with emotional regulation, resilience and positive affect in both second and third trimester of pregnancy in 152 pregnant women(102).

Sleep and diet have been also associated in recent studies: food choices might influence sleep quality(103). Cross-sectional studies have postulated that diets with high intake of fruits and vegetables and low in saturated fatty acids, such as the Mediterranean diet, could be beneficial for sleep quality in the general adult population(104,105). Regarding the potential effects of the Mediterranean diet on sleep quality in pregnant women, in the GESTAFIT trial in Spain, authors conclude that the group with the highest adherence to Mediterranean diet, a greater intake of fruits and olive oil and a lower intake of red meat had better sleep quality during pregnancy, assessed with the PQSI at second and third trimester of gestation(106).

3.5.2. The effect of a Mindfulness intervention program

Mindfulness is the mental state achieved by focusing one's awareness on the present moment while calmly acknowledging one's feelings, thoughts and bodily sensations(107). Mindfulness meditation and other mind–body therapies have emerged as helpful adjuncts for stress-related noncommunicable diseases(108). In order to assess the evidence of meditation programs to improve stress-related outcomes, in 2014, Goyal *et al.* published a systematic review and metanalysis of 47 randomized controlled trials showing effectiveness in reducing negative outcomes such as stress or depression and chronic pain(109). Mindfulness-based stress reduction (MBSR) program is one of the most well-described structured programs and has extensively been used in medical research(110). It was first launch by the University of Massachusetts Medical School and minor modifications can be introduced to adapt the program to pregnant women.

In pregnancy, several small studies have reported that MBSR reduced perceived stress and anxiety(111). In a randomized controlled trial in 2019, Pan *et al.* reported benefits of a mindfulness-based intervention program in self-perceived stress and depression, suggesting that the program could provide acceptable and long-term benefits in pregnant and postpartum women(112). Moreover, in 2021, Crovetto *et al.* reported in a randomized controlled trial, benefits in the reduction of small for gestational age newborns in high risk pregnancies when applying an intervention based in MBSR program(101).

3.6. Maternal microbiota

The term microbiota describes the living microorganisms found in a defined environment, such as oral, vaginal or gut microbiota(113). It has become more evident the importance of the gut microbiota for human health since specific links between alterations of microbiota composition (*dysbiosis*) and several pathological conditions such as cancers, diabetes, and neurological disorders have been described(113). Table 1 reflects the composition of human microbiota(113).

Human Microbiota Composition(113)				
Oral Cavity	**Respiratory Tract**	**Gut**	**Vagina**	**Skin**
Firmicutes Proteobacteria Bacteroidetes Actinobacteria Fusobacteria	Actinobacteria Bacteroidetes Firmicutes Proteobacteria	6 phyla: Firmicutes Bacteroidetes Actinobacteria Proteobacteria Fusobacteria Verrucomicrobia *(Major types: Firmicutes and Bacteroidetes) Fungi: Candida Saccharomyces Malassezia Cladosporium Additionally: viruses, phages, and archaea (mainly M. smithii)	Lactobacillus	Actinobacteria Bacteroidetes Cyanobacteria Firmicutes Proteobacteria

Table 1. Human Microbiota composition(113). Source: own elaboration.

3.6.1. Gut microbiota

Gut microbiota is considered the most significant microbiota in our body in maintaining our health(113) through fermentation of food, protection against pathogens, stimulating immune response, and vitamin production(114).

The gut-brain axis consists of the relationship between the central and enteric nervous system(115). In the 1980s, the development of brain imaging, brought light to the bidirectional communication between this axis(116). Recent advances in research have described the importance of gut microbiota as a key regulator of the gut-brain axis(113,115), constituting a new concept: the microbiota-gut-brain axis, linking microbiota, gut function and cognitive/emotional brain centers(117). This interaction between microbiota and the gut-brain axis appears to be also bidirectional (figure 2), namely through signaling from gut-microbiota to brain and from brain to gut-microbiota by means of neural, endocrine, immune, and humoral links(115).

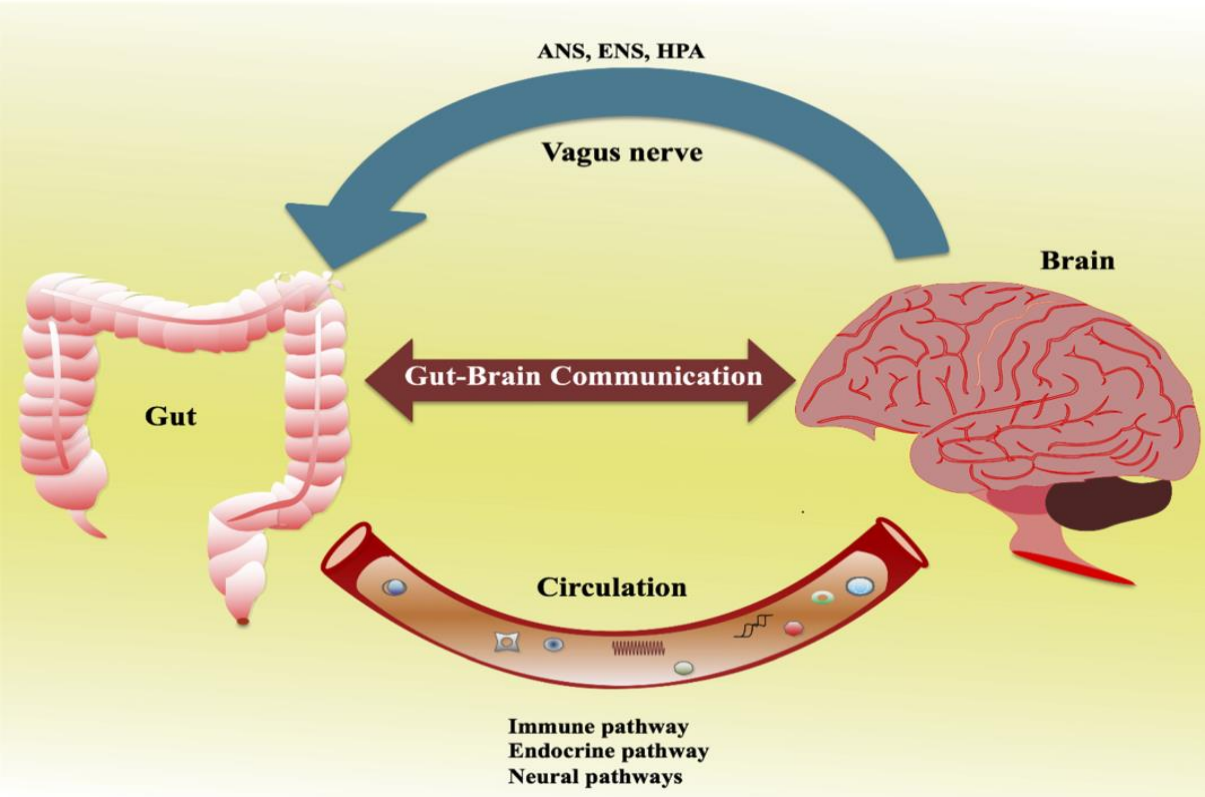

Figure 2: The Gut-Brain Axis. Authorship: Suganya, Kanmani, and Byung-Soo Koo(118) ©2020 *Creative Commons License.*

3.6.2. Respiratory tract microbiota

The upper respiratory tract is the major portal of entry for infectious droplets or aerosol-transmitted microorganisms. The barrier function of its mucosa and the regulation of the immune response are modulated by the microbiota.

Evidence suggests that dysbiosis of the upper respiratory tract (nose and nasopharynx) microbiota modulates the hosts susceptibility to pathological conditions, such as acute respiratory tract infections(119)

Pregnancy is a unique physiological state in which all body systems participate, including hormonal, immune and metabolic pathways(120). Although changes in gut microbiota over pregnancy have been reported(121), other human microbiota with potential physiological effects , such as the respiratory tract microbiota, have been poorly studied

3.6.3. Vaginal microbiota

The vaginal microbiota is believed to play a crucial role in protecting the host from pathogenic colonizations, such as vulvovaginal candidiasis, sexually transmitted infections and even urinary tract infections(113). In the healthy-state vagina, Lactobacillus-dominated community is likely to be observed (*L. crispatus, L. gasseri, L. iners*), together with a low diversity of anaerobic bacteria (*Gardnerella vaginalis, Prevotella spp., Mobiluncus spp., Ureaplasma urealyticum, and Mycoplasma hominis*) and a balanced vaginal immune system (e.g. pro-inflammatory and anti-inflammatory cytokines)(113,122).

3.6.4. Mental health and microbiota

Stress has effects on the gastrointestinal tract and can lead to long-term changes in the gut microbiota(123). Chronic stress can induce *dysbiosis* and enhance bacterial wall adherence, whereas the interaction between host and microbiota can modulate the neuroimmune-endocrine system. Moreover, stress has been shown to reduce microbial diversity(123). Preclinical and clinical evidence suggest that the gastrointestinal microbiota influence mood and behavior, including depression, anxiety, and stress(94). The exact mechanisms by which microbes affect mood in humans are yet to be understood, but multiple studies have demonstrated that the gut microbiota plays an important role in mental health(94). In a study in rodents, depression-like behaviors were observed in mice who underwent a fecal microbiota transplantation of patients with major depression(124). When comparing the gut microbiota of depressed individuals to healthy individuals, differences in the microbiota composition have been observed have been compared to healthy individuals(125). However, to truly understand the mechanisms and impact that the gut microbiota have on mood, additional clinical trials are needed(94).

The important microorganisms for the colonization of the children's microbiota mainly originate from the mother(126). It is true that the principal colonization of the neonatal gut starts at delivery but recent research demonstrates that microbiota origins already from fetal life(126,127), suggesting the existence of a mother-placenta-fetus axis which permits the circulation of maternal bacteria to the fetus, being the maternal microbiota the main origin of the fetal and neonatal microbiota not only through delivery, but also through the placenta(126,127).

Changes in vaginal(128) and gut(129) microbiota during pregnancy have been demonstrated. Moreover, in a human study conducted on 70 mothers who provided a late pregnancy stool sample, authors report changes in the infant microbiota of the mothers with higher rates of self-reported stress and anxiety(130). Therefore, maternal microbiota may be involved in the neonatal development with implications for the future adult health(131), including the risk of obesity, metabolic syndrome, diabetes, and allergy-related problems(132,133).

Even though gestational and perinatal factors that shape the maternal microbiota are yet to be truly defined, lifestyles, especially diet, have resulted to be critical factors in modulating the microbiota and, therefore, the gut-brain axis(113). For example, the Mediterranean diet has been shown to significantly reduce the occurrence of neurovegetative disorders, psychiatric conditions, cancer, and cardiovascular diseases(88–91,134). Mediterranean diet also showed a reduction in the risk of depression as stated above(99,135).

Thus, strategies targeting maternal microbiota during pregnancy may offer new directions for preventive and therapeutic applications impacting both maternal and infant health at the same time. A few studies reported influences of maternal lifestyle during pregnancy on microbiota composition, but all of them came from observational studies without applying structured interventions(126,130).

3.7. Rational of the study

To sum up, there is growing evidence that maternal psychological status can be compromised during pregnancy, as it consists of a unique potentially challenging emotional

time for many women. Many studies report that compromised maternal mental health affects maternal and pregnancy outcomes but also the fetal environment and this prenatal environment is important for the normal fetal development.

With our research, we wanted to describe the maternal mental health of our pregnant population and identify potential risk factors for a compromised status. Moreover, we wanted to see how the maternal environment in different situations could have an impact on maternal mental status and well-being and on pregnancy outcome. In this context, we tested several situations:

- A negative maternal environment: The COVID-19 pandemic and lockdown.

- A positive maternal environment: an intervention during pregnancy based on Mediterranean diet and/or MBSR.

Additionally, in both situations we wanted to evaluate if these environmental conditions could also affect the maternal microbiota and if the impact on maternal mental status could be mediated by these changes in the maternal microbiota.

4. Hypothesis

The **main hypothesis** is that maternal environment during pregnancy can have an impact on maternal mental health and sleep quality.

Specific hypothesis are as follows:

Hypothesis 1. Pregnancy itself has a unique impact on maternal mental health.

Hypothesis 2. COVID-19 pandemic and lockdown had a negative impact on maternal mental health.

Hypothesis 3. Specific lifestyle interventions during pregnancy have a positive impact on maternal mental health.

Hypothesis 4. Changes in the composition of the maternal respiratory tract microbiota can be seen in SARS-CoV-2 infection.

Hypothesis 5. The maternal microbiota could be considered a possible mediator of a biological effect of maternal brain status: changes in its composition can be seen in lifestyle interventions.

5. Objectives

The **main objective** is to evaluate the effects of different maternal environments during pregnancy on maternal mental status and sleep quality.

Specific objectives are as follows:

Objective 1. To describe maternal stress, anxiety, well-being and sleep quality during pregnancy in general population and to identify potential risk factors for distress.

Objective 2. To evaluate the effect of the COVID-19 pandemic and lockdown on maternal mental health and to identify potential risk factors.

Objective 3. To evaluate the impact of a Mediterranean diet-based program during pregnancy on maternal mental health.

Objective 4. To evaluate the changes on maternal respiratory tract microbiota during COVID-19 pandemic.

Objective 5. To evaluate the changes on maternal gut and vaginal microbiota during an intervention based on Mediterranean diet and Mindfulness Based Stress Reduction techniques, and its correlation with maternal mental health during pregnancy, in order to propose the maternal microbiota as a potential biomarker of stress-related problems.

6. Materials, methods and results

STUDY 1

Maternal Anxiety, Stress, Well-being, and Sleep Quality in pregnant women throughout gestation.

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Maternal Stress, Anxiety, Well-Being, and Sleep Quality in Pregnant Women throughout Gestation

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Abstract: Background: Maternal stress, anxiety, well-being, and sleep quality during pregnancy have been described as influencing factors during pregnancy. Aim: We aimed to describe maternal stress, anxiety, well-being, and sleep quality in pregnant women throughout gestation and their related factors. Methods: A prospective study including pregnant women attending BCNatal, in Barcelona, Spain (n = 630). Maternal stress and anxiety were assessed by the Perceived Stress Scale (PSS) and State-Trait Anxiety Inventory (STAI)-validated questionnaires. Maternal well-being was assessed using the World Health Organization Well-Being Index Questionnaire (WHO-5), and sleep quality was assessed using the Pittsburgh Sleep Quality Index Questionnaire (PSQI). All questionnaires were obtained twice during the second and third trimester of pregnancy. A multivariate analysis was conducted to assess factors related to higher maternal stress and anxiety and worse well-being and sleep quality. Results: High levels of maternal stress were reported in 23.1% of participants at the end of pregnancy, with maternal age <40 years (OR 2.02; 95% CI 1.08–3.81, p = 0.03), non-white ethnicity (OR 2.09; 95% CI 1.19–4.02, p = 0.01), and non-university studies (OR 1.86; 95% CI 1.08–3.19, p = 0.02) being the parameters mostly associated with it. A total of 20.7% of women had high levels of anxiety in the third trimester and the presence of psychiatric disorders (OR 3.62; 95% CI 1.34–9.78, p = 0.01) and non-university studies (OR 1.70; 95% CI 1.11–2.59, p = 0.01) provided a significant contribution to high anxiety at multivariate analysis. Poor maternal well-being was observed in 26.5% of women and a significant contribution was provided by the presence of psychiatric disorders (OR 2.96; 95% CI 1.07–8.25, p = 0.04) and non-university studies (OR 1.74; 95% CI 1.10–2.74, p = 0.02). Finally, less sleep quality was observed at the end of pregnancy (p < 0.001), with 81.1% of women reporting poor sleep quality. Conclusion: Maternal stress and anxiety, compromised maternal well-being, and sleep quality disturbances are prevalent throughout pregnancy. Anxiety and compromised sleep quality may increase over gestation. The screening of these conditions at different stages of pregnancy and awareness of the associated risk factors can help to identify women at potential risk.

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1. Introduction

According to the World Health Organization (WHO), health is a "state of complete physical, mental, and social well-being, and not merely the absence of disease or infirmity". Therefore, mental health, defined by the WHO as a "state of mental well-being that enables people to cope with the stresses of life, realize their abilities, learn well and work well, and contribute to their community", is as fundamental as physical health in the achievement of positive overall wellness in an individual [1].

Stress, anxiety, compromised mental well-being, and sleep quality are fundamental and interconnected aspects of mental health. They can impact each other and together contribute to a general state of emotional and mental wellness. Mental stress can be medically understood as the 'individual's perception of a stimulus as overwhelming' which results in a response and a transformed state [2]. Anxiety is defined by the American Psychological Association as "an emotion characterized by feelings of tension, worried thoughts, and physical changes like increased blood pressure." Both stress and anxiety are emotional responses. Stress is usually precipitated by an external factor, whereas anxiety is defined by the persistence of excessive worries even in the absence of a stressor. Well-being is broadly defined as 'the quality and state of a person's life' [3] and consists of two components: feeling healthy and relatively robust and being able to carry out one's job and other tasks satisfactorily [4]. Finally, sleep quality is defined as an individual's level of satisfaction with all aspects of the sleep experience [5]. Sleep quality is highly dependent on the person's general well-being.

Maternal mental stress, anxiety, compromised well-being, and sleep quality have been associated with several adverse pregnancy outcomes such as preterm birth (PTB) [6–12], low birthweight (LBW) [7,13–15], gestational diabetes (GD) [16,17], labor complications [12,18–21], or hypertension and preeclampsia (PE) [22,23]. Moreover, maternal stress has been demonstrated to be a prenatal programming factor that affects the fetal neurodevelopment [24] and could compromise the socioemotional competencies in childhood that are the foundation for future well-being [24].

Mental stress, anxiety, compromised well-being, and sleep disturbances are common during pregnancy. Around 20% of pregnant women could experience excessive concern regarding future events in pregnancy under normal circumstances [4]. Up to 70% of pregnant women report symptoms of stress and anxiety during pregnancy, with between 10% and 16% of them fulfilling the criteria for a major depressive disorder [25,26]. While the real prevalence of antenatal psychosocial stress is still unclear [27], in a 2003 study, Rondó et al. found high stress in 22–25% of pregnant women during the three trimesters of pregnancy [7]. In a meta-analysis of 102 studies involving 221,974 women, Dennis et al. found that the prevalence rate for self-reported anxiety symptoms in the first trimester was 18.2% and 24.6% in the third trimester [28]. These percentages decreased when employing diagnostic interviews: the prevalence rate for any anxiety disorder during the first trimester was 18% and 15% in the final two trimesters of pregnancy [28]. However, we can speculate that the symptoms of depression can overlap with some normal feelings during pregnancy, which could explain such high percentages and the disparity found among studies [26]. There is no clear evidence of the prevalence of compromised well-being during pregnancy. A highly variable prevalence of poor sleep quality in pregnant women has also been reported, ranging from 17% to 76% [29]. This disparity could be due to dissimilar sample compositions and different methods and timings of assessments [30]. Moreover, some authors have even postulated the possibility that the previously validated cut-off values for sleep questionnaires in the general population may not be valid in pregnancy, thus requiring a higher score [30].

Different risk factors for antenatal mood disorders have been postulated in the previously published literature. Sociodemographic variables such as age have been considered in multiple studies with inconsistent findings among them [31,32]. Other sociodemographic variables considered in the previous literature are maternal socioeconomic status and educational level: in a 2010 systematic review, Lancaster et al. found a small association between low educational level and depression symptoms that could not be demonstrated in the multivariate analyses [33]. Later, Biaggi et al. found low maternal educational level to be associated with anxiety and depressive symptoms [32]. As for ethnicity, socioeconomic status, employment, an unfavorable socioeconomic situation, unemployment, and belonging to a minority ethnic group are associated with depression in several studies [31,32,34] but inconsistent results are described in others [32,33]. On the other hand, other factors such as smoking, alcohol intake, and drug abuse showed inconsistent findings in their association with depression and sleep quality [29,32–34]. A personal medical history of anxiety and depression has strongly been associated with perinatal depression [31–34]. Other studies suggest an association between previous obstetric history, like previous abortions or pregnancy complications, with depressive symptoms and poor sleep quality [29,31,32] but also with inconsistent findings [33]. A complex multifactorial origin for the etiology of these conditions could be a possible explanation for such different results reported in the literature [33].

Despite the high prevalence of these antenatal negative affective states and their impact on pregnancy, it is still unclear if they worsened during pregnancy and what the potential risk factors for these conditions are during pregnancy.

The aim of this study was to determine maternal stress, anxiety, well-being, and sleep quality across different stages of pregnancy and to identify related risk factors.

2. Materials and Methods

2.1. Study Design and Participants

A prospective study was carried out at BCNatal (Hospital Clinic and Hospital Sant Joan de Déu), a large referral center for maternal-fetal and neonatal medicine in Barcelona, Spain. Inclusion criteria were pregnant women with a singleton fetus who attended our center for their second trimester scan (19–23 weeks of gestation), and who were able to respond to maternal stress, anxiety, well-being, and sleep quality validated questionnaires. The exclusion criteria for the study are as follows: maternal mental retardation or other mental or psychiatric disorders that raise doubts regarding the patient's real willingness to participate in the study and the impossibility of completing questionnaires or other procedures in the study, congenital infections, fetal anomalies including chromosomal abnormalities or structural malformations detected by ultrasound prenatally, and neonatal abnormalities diagnosed after birth. The study was approved by the hospital ethical committee (HCB-2016-0830 and HCB/2020/0209) and written informed consent was obtained from all participants.

2.2. Study Aims

The main aim of the study was to evaluate maternal stress, anxiety, well-being, and sleep quality at two moments during pregnancy, assessed using four different validated questionnaires: the Perceived Stress Scale (PSS) [35] and State-Trait Anxiety Inventory (STAI) [36] for maternal stress and anxiety, respectively, the World Health Organization Well-Being Index Questionnaire (WHO-5 Index) for maternal well-being [37], and the Pittsburgh Sleep Quality Index (PSQI) [38] for sleep quality.

The secondary aim was to evaluate maternal and pregnancy factors acting as potential risk factors for increased maternal stress and anxiety, poorer maternal well-being status, and poorer sleep quality during gestation.

2.3. Data Collection

All questionnaires were completed twice during pregnancy: at recruitment of the study population in their second trimester of pregnancy (19–23 weeks of gestation) and again at the end of the third trimester of pregnancy (34–36 weeks of gestation).

The Perceived Stress Scale was designed to measure "the degree to which individuals appraise situations in their lives as stressful" [35]. It is a brief scale, consisting of only 14 items evaluating stress within the last 8 weeks. PSS scores are obtained by reversing responses to the 4 positively stated items (items 4, 5, 7, and 8) and then adding across all scale items. It is not a diagnostic instrument; therefore, there are no cut-offs for classification of the stress, but it gives a comparison instrument between people [39]. The higher stress group in this cohort was considered the 75th percentile at the first evaluation (19–23 weeks of gestation).

The STAI questionnaire consists of two subscales: the State Anxiety Scale (STAI-S), which evaluates the current state of anxiety, and the Trait Anxiety Scale (STAI-T), which evaluates individual aspects of "anxiety proneness". The STAI has 40 items, 20 items allocated to each of the S-State and T-Trait subscales. The range of scores for each subtest is 20–80, the higher indicating greater anxiety [40,41]. The higher stress group in this cohort was considered the 75th percentile at the first evaluation (19–23 weeks of gestation).

The WHO-5 consists of a five-item scale and it is used to rate quality of life and psychological well-being, according to the participant's feelings within the last 15 days. The raw score ranges from 0 to 25: 0 representing worst possible and 25 representing best possible quality of life. Following total scores, standardized scores (0–100) are calculated. Women were classified according to their well-being status as with a poor ($\leq$52) or favorable (>52) WHO-5 score [42].

The PSQI assesses sleep quality and disturbances over a monthly interval. It contains 19 self-rated questions which are combined to form 7 component scores: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction. Each of these components has a range of 0–3 points (where 0 means no difficulty and 3 indicates severe difficulty). The 7 component scores are added to give a global score, with a range of 0–21 points, 0 indicating no difficulty and 21 indicating severe difficulties in all areas. A global PSQI score greater than 5 defines poor sleep quality [38].

Baseline and socioeconomic characteristics, such as maternal age, ethnicity, educational level, or pre-pregnancy body mass index (BMI) were obtained from a structured questionnaire. Medical and obstetric history were obtained from the medical records at recruitment.

2.4. Statistical Analysis

For the first aim, the analysis was based on the scores of PSS, STAI-S, STAI-T, WHO-5, and PSQI-validated questionnaires. Continuous variables were assessed for normality using the Shapiro–Wilk's test. Normally distributed variables were compared using a t-test and expressed as mean and standard deviation (SD). Non-normally distributed variables were compared using the U–Mann–Whitney test and expressed as the median and interquartile range (IQR). Categorical variables were compared using χ^2 or Fisher's exact test where appropriate. To study the correlation of the different tests, Pearson correlation analyses were performed. For the secondary outcomes, logistic regression analysis with forward stepwise selection was performed to assess the association between maternal higher stress (>p75) (PSS, STAI-S, STAI-T), poor well-being ($\leq$52 WHO-5), and lower sleep quality (>5 PSQI), with potential maternal risk factors at final evaluation (34–36 weeks of gestation). A multivariate analysis was performed for the variables found to have a significant effect in bivariate analyses. The odds ratio (OR) and a 95% confidence interval (95% CI) were calculated. A p-value < 0.05 was considered statistically significant. The analysis was performed using SPSS v26 (New York, NY, USA).

3. Results

3.1. Study Population

A total of 630 women were recruited in the second trimester at a median [IQR] gestational age of 20 weeks [20,21]). The majority of women (n = 497, 79.3%) were of white ethnicity and with university studies (n = 427, 68%). Baseline characteristics of the study population are shown in Table 1. Regarding their medical history, 2.7% of women (n = 17) had psychiatric disorders requiring therapy, 5.6% (n = 35) had thyroid disorders, and 7.8% (n = 49) had a BMI $\geq$ 30.

Table 1. Baseline characteristics of participants included in the study (n = 630).

Characteristics	Total Cohort n = 630
Age at recruitment (years)	35.8 (32.2–38.7)
Ethnicity	
White	497 (79.3%)
Latin	98 (15.6%)
Afro-American	6 (1%)
Asian	16 (2.6%)
Others	10 (1.6%)
Low socioeconomic status [(a)]	25 (4%)
Study class	
Primary	25 (4%)
Secondary	176 (28%)
University	427 (68%)
BMI before pregnancy (Kg/m^2)	23.4 (4.1)
Medical history	
Autoimmune disease	64 (10.2%)
Obesity (BMI $\geq$ 30)	49 (7.8%)
Thyroid disorders	35 (5.6%)
Chronic hypertension	18 (2.9%)
Psychiatric disorders [(b)]	17 (2.7%)
Diabetes mellitus	14 (2.2%)
Chronic kidney disease	8 (1.3%)
Obstetric history	
Nulliparous	393 (62.4%)
Previous preeclampsia	17 (2.7%)
Previous stillbirth	5 (0.8%)
Use of assisted reproductive technologies	121 (19.2%)
Cigarette smoking during pregnancy	81 (12.9%)
Alcohol intake during pregnancy	14 (2.2%)
Drug consumption during pregnancy	15 (2.4%)
Sports practice during pregnancy	103 (16.3%)
Yoga or Pilates during pregnancy	141 (22.4%)

BMI: body-mass index. Data are expressed as median (IQR) or mean (SD) or n (%). [a] Low socioeconomic status: low (never worked or unemployed >2 years). [b] Psychiatric disorders: requiring therapy for psychiatric disorder.

3.2. Stress, Anxiety, Well-Being, and Sleep Quality throughout Pregnancy

The median [IQR] scores of the PSS at the second trimester evaluation was 16 (11–22), and it did not change during pregnancy, as reported in Table 2 and Figure 1A. No changes during gestation were found for STAI-T and for the well-being evaluation (WHO-5) (see Table 2 and Figure 1B,C). On the contrary, an increasing score during the third trimester was observed for the STAI-S ($p < 0.001$) and PSQI questionnaires ($p < 0.001$) (see Table 2 and Figure 1D,E).

The correlation between the final results of the stress and anxiety tests was calculated by the Pearson correlation coefficient, which showed a significative positive strong correlation between the levels of stress and anxiety (PSS vs. STAI-S, r = 0.72, $p < 0.001$; PSS vs. STAI-T, r = 0.69, $p < 0.001$; STAI-T vs. STAI-S, r = 0.75, $p < 0.001$). A significative

negative moderate correlation was observed between WHO-5 and the stress and anxiety tests, highlighting poorer mental well-being in relation to higher levels of anxiety and stress (WHO-5 vs. PSS, r = −0.58, $p < 0.001$; WHO-5 vs. STAI-S, r = −0.63, $p < 0.001$; WHO-5 vs. STAI-T, r = −0.65, $p < 0.001$). Finally, the correlation found between sleep quality and stress, anxiety, and mental well-being was low (PSQI vs. STAI-S, r = 0.31, $p < 0.001$; PSQI vs. STAI-T, r = 0.34, $p < 0.001$; PSQI vs. PSS, r = 0.33, $p < 0.001$; PSQI vs. WHO-5, r = 0.40, $p < 0.001$).

Table 2. Stress, anxiety, and sleep quality of women included in the study at second and third trimester of pregnancy (n = 630).

Characteristics	2nd Trimester n = 630	3rd Trimester n = 630	*p* Value
Perceived stress scale score	16 (11–22)	16 (12–23)	0.07
State-trait Anxiety Inventory (anxiety)	13 (8–18)	14 (9–20)	<0.001
State-trait Anxiety Inventory (personality)	14 (10–21)	15 (10–21)	0.88
Five well-being Index	68 (56–76)	64 (52–76)	0.81
Pittsburg quality sleep index	7 (5–8.5)	8 (9–10)	<0.001

Data are expressed as median (IQR).

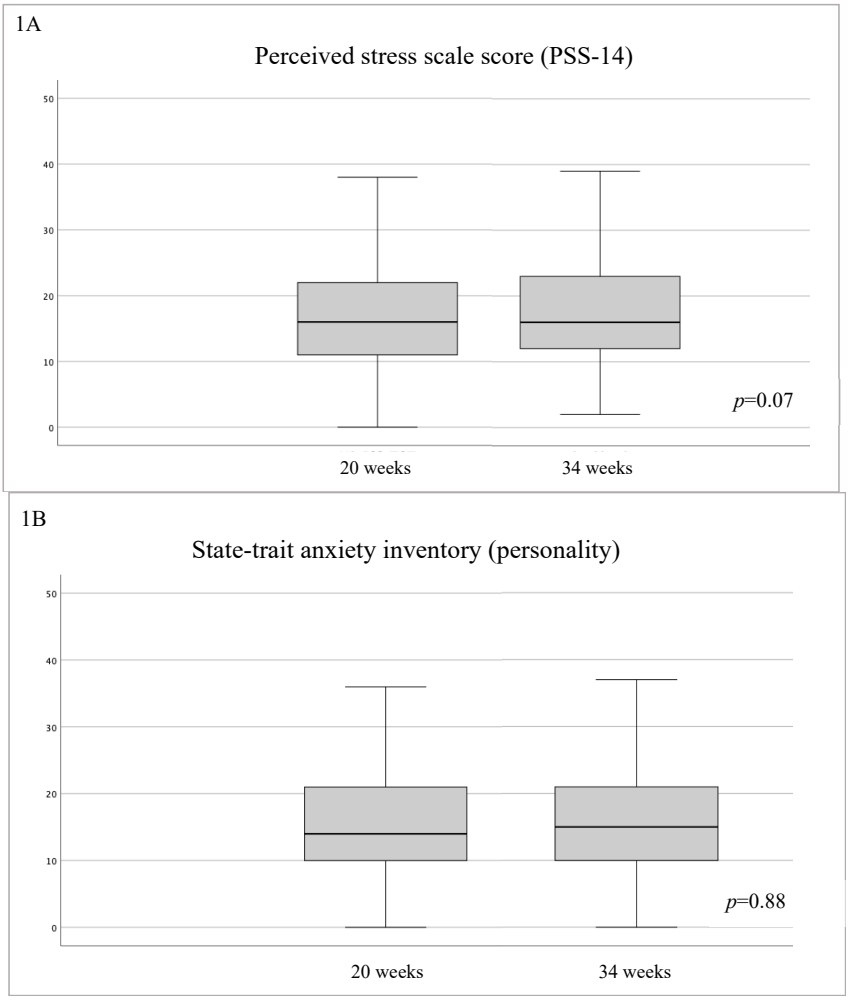

Figure 1. *Cont.*

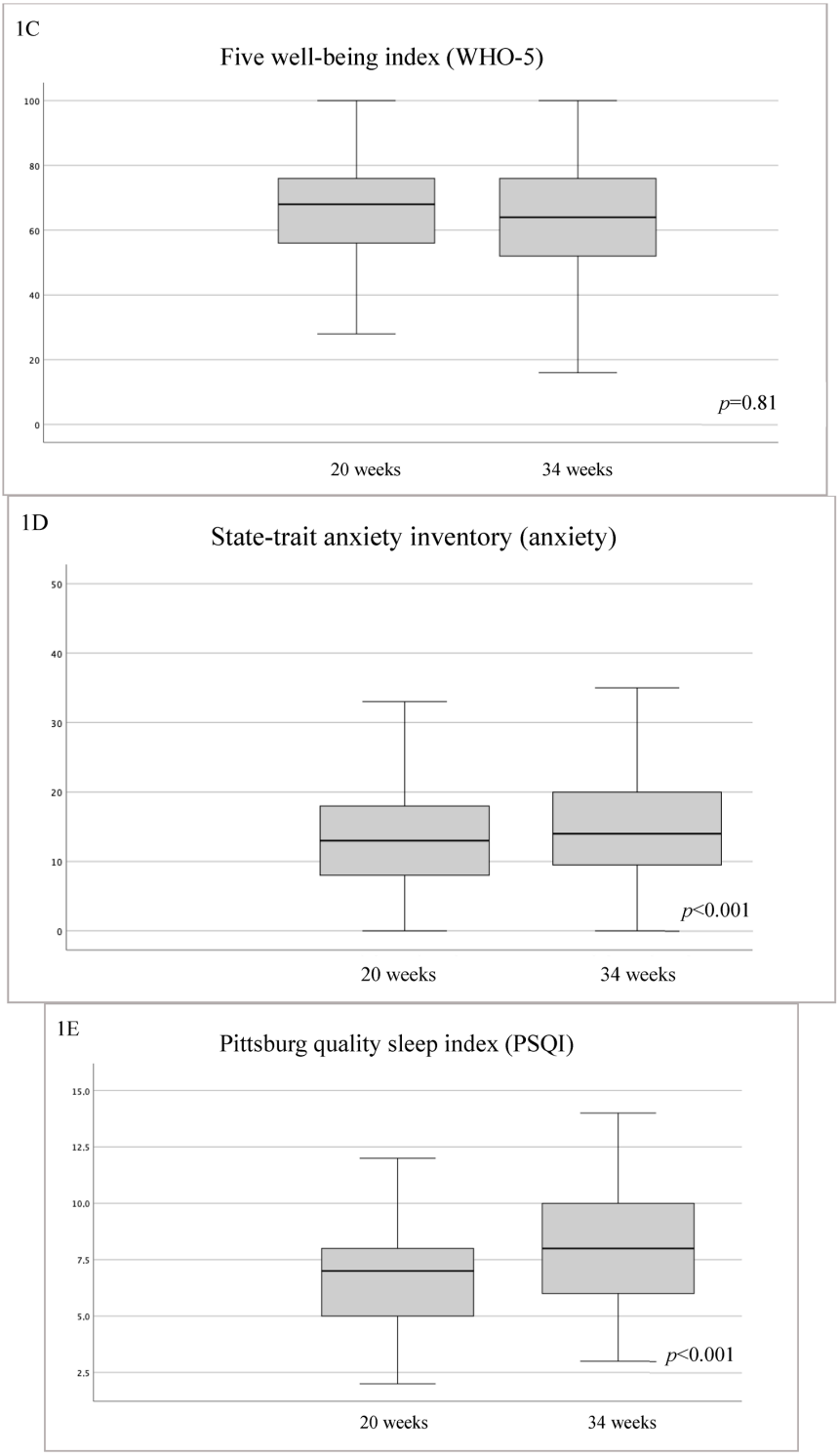

Figure 1. (**A**) Evolution in PSS-14 test score at baseline (20 weeks) and at the end of gestation (34 weeks). The median (IQR) scores of the PSS at second trimester evaluation was 16 (11–22) and it did not change during pregnancy. (**B**) Evolution in STAI-T test score at baseline (20 weeks) and at the end of gestation (34 weeks). No changes during gestation were found. (**C**) Evolution in WHO-5 test score at baseline (20 weeks) and at the end of gestation (34 weeks). No changes during gestation were found. (**D**) Evolution in STAI-S test score at baseline (20 weeks) and at the end of gestation (34 weeks). An increased score during the third trimester was observed. (**E**) Evolution in PSQI test score at baseline (20 weeks) and at the end of gestation (34 weeks). An increased score during the third trimester was observed.

3.3. Maternal Stress and Anxiety

High levels of maternal PSS were reported in 115 women (23.1%) at the end of pregnancy. At multivariate analysis, a significant contribution to this condition was provided by maternal age <40 years (OR 2.02; 95% CI 1.08–3.81, $p = 0.03$), non-white ethnicity (OR 2.09; 95% CI 1.19–4.02, $p = 0.01$), and non-university studies (OR 1.86; 95% CI 1.08–3.19, $p = 0.02$). Details are reported in Table 3.

Table 3. Univariate and multivariate analysis of factors associated with a poor maternal PSS-14 questionnaire.

Characteristics	Univariate Analysis		Multivariate Analysis		
	OR (95% CI)	*p* Value	OR (95% CI)	*p* Value	Beta Coefficient
Maternal age < 40 years	2.24 (1.21–4.15)	0.01	2.01 (1.08–3.81)	0.03	0.705
Ethnicity					
White	0.42 (0.26–0.69)	<0.001			
Non-white	2.36 (1.45–3.83)	<0.001	2.19 (1.19–4.02)	0.01	0.786
Low socioeconomic status [a]	1.82 (0.75–4.41)	0.18			
Study class					
Primary or secondary	2.19 (1.43–3.39)	<0.001	1.86 (1.08–3.19)	0.02	0.620
University	0.46 (0.30–0.70)	<0.001			
Medical history					
Obesity (BMI $\geq$ 30)	1.27 (0.633–2.56)	0.5			
Diabetes mellitus	1.34 (0.41–4.35)	1.34			
Autoimmune disease	1.15 (0.63–2.12)	0.65			
Thyroid disorders	0.53 (0.20–1.41)	0.2			
Psychiatric disorders [b]	1.85 (0.67–5.13)	0.233			
Chronic hypertension	0.66 (0.19–2.3)	0.51			
Obstetric history					
Nulliparous	1.11 (0.73–1.71)	0.62			
Previous preeclampsia	3 (1.17–8.22)	0.02	2.7 (0.98–7.46)	0.06	0.993
Use of assisted reproductive technologies	0.58 (0.34–1.01)	0.06			
Cigarette smoking during pregnancy	0.93 (0.52–1.67)	0.81			
Alcohol intake during pregnancy	1.18 (0.74–1.87)	0.5			
Drug consumption during pregnancy	1.89 (0.62–5.75)	0.26			
Yoga or Pilates during pregnancy	0.73 (0.43–1.23)	0.24			
Constant			−2.204		
Data are expressed as n (%)					

PSS: Perceived Stress Scale; OR: Odds Ratio; CI: confidence interval; BMI: body-mass index. [a] Low socioeconomic status: low (never worked or unemployed >2 years). [b] Psychiatric disorders: requiring therapy for psychiatric disorder.

According to the STAI questionnaire (anxiety, STAI-S), 129 women (20.7%) had high levels of anxiety in the third trimester. In these women, a significant contribution to multivariate analysis was provided by the presence of psychiatric disorders (OR 3.62; 95% CI 1.34–9.78, $p = 0.01$), and non-university studies (OR 1.70; 95% CI 1.11–2.59, $p = 0.01$). Details are reported in Table 4.

According to the STAI-T personality questionnaire, 116 women (23.6%) ended pregnancy with a high anxiety trait level. In the multivariate analysis, a significant contribution to this condition was provided by maternal age <40 years (OR 2.07; 95% CI 1.11–3.88, $p = 0.02$) and preeclampsia in a previous pregnancy (OR 2.9; 95% CI 1.03–8.2, $p = 0.04$). Details are reported in Table 5.

3.4. Maternal Well-Being

Poor maternal well-being (WHO-5 score $\leq$52) was observed in 131 women (26.5%) in the 3rd trimester assessment. Significant contribution to a low maternal well-being was provided by the presence of psychiatric disorders (OR 2.96; 95% CI 1.07–8.25, $p = 0.04$), and non-university studies (OR 1.74; 95% CI 1.10–2.74, $p = 0.02$). Details are reported in Table 6.

Table 4. Univariate and multivariate analysis of factors associated with a poor maternal STAI anxiety questionnaire.

Characteristics	Univariate Analysis		Multivariate Analysis		
	OR (95% CI)	*p* Value	OR (95% CI)	*p* Value	Beta Coefficient
Maternal age < 40 years	1.69 (0.92–3.12)	0.09			
Ethnicity					
White	0.7 (0.44–1.1)	0.12			
Non-white	1.43 (0.91–2.26)	0.12			
Low socioeconomic status [(a)]	0.76 (0.26–2.27)	0.63			
Study class					
Primary or secondary	1.75 (1.17–2.61)	0.01	1.70 (1.11–2.59)	0.01	0.529
University	0.57 (0.38–0.85)	0.01			
Medical history					
Obesity (BMI $\geq$ 30)	1.01 (0.49–2.09)	0.97			
Diabetes mellitus	1.05 (0.29–3.8)	0.95			
Autoimmune disease	1.81 (1.02–3.23)	0.04	1.56 (0.84–2.89)	0.16	0.446
Thyroid disorders	2.1 (1.02–4.34)	0.04	1.81 (0.84–3.90)	0.13	0.595
Psychiatric disorders [(b)]	3.56 (1.35–9.42)	0.01	3.62 (1.34–9.78)	0.01	1288
Chronic hypertension	0.76 (0.22–2.67)	0.67			
Chronic kidney disease	1.28 (0.25–6.42)	0.76			
Obstetric history					
Nulliparous	1.43 (0.96–2.12)	0.07			
Previous preeclampsia	1.77 (0.6–5.19)	0.3			
Previous stillbirth	0.96 (0.11–8.63)	0.97			
Use of assisted reproductive technologies	0.64 (0.37–1.01)	0.11			
Cigarette smoking during pregnancy	1.13 (0.64–2)	0.67			
Alcohol intake during pregnancy	0.92 (0.59–1.43)	0.72			
Drugs consumption during pregnancy	0.96 (0.27–3.47)	0.96			
Yoga or Pilates during pregnancy	0.5 (0.29–0.85)	0.01	0.62 (0.35–1.1)	0.1	−1.059
Constant			−1.586		
Data are expressed as n (%)					

STAI: State-Trait Anxiety Inventory; OR: Odds Ratio; CI: confidence interval; BMI: Body-mass index. [a] Low socioeconomic status: low (never worked or unemployed >2 years). [b] Psychiatric disorders: requiring therapy for psychiatric disorder.

Table 5. Univariate and multivariate analysis of factors associated with a poor maternal STAI personality questionnaire.

Characteristics	Univariate Analysis		Multivariate Analysis		
	OR (95% CI)	*p* Value	OR (95% CI)	*p* Value	Beta Coefficient
Maternal age < 40 years	2.28 (1.23–4.23)	0.01	2.07 (1.11–3.88)	0.02	0.729
Ethnicity					
White	0.54 (0.33–9.86)	0.02			
Non-white	1.85 (1.13–3.03)	0.02	1.55 (0.83–2.92)	0.17	−0.440
Low socioeconomic status [(a)]	1.22 (0.47–3.19)	0.68			
Study class					
Primary or secondary	2.09 (1.36–3.22)	0.01	1.42 (0.81–2.49)	0.22	0.350
University	0.48 (0.31–7.36)	0.01			
Medical history					
Obesity (BMI $\geq$ 30)	0.95 (0.45–1.98)	0.88			
Diabetes mellitus	1.83 (0.60–5.58)	0.28			
Autoimmune disease	1.26 (0.69–2.3)	0.45			
Thyroid disorders	0.96 (0.42–2.16)	0.91			
Psychiatric disorders [(b)]	2.34 (0.87–6.30)	0.09			
Chronic hypertension	0.64 (0.18–2.24)	0.48			
Chronic kidney disease	0.46 (0.05–3.75)	0.47			

Table 5. *Cont.*

Characteristics	Univariate Analysis		Multivariate Analysis		
	OR (95% CI)	*p* Value	OR (95% CI)	*p* Value	Beta Coefficient
Obstetric history					
Nulliparous	1.16 (0.76–1.77)	0.50			
Previous preeclampsia	3.4 (1.25–9.27)	0.01	2.9 (1.03–8.2)	0.04	1.069
Previous stillbirth	0.81 (0.09–7.29)	0.85			
Use of assisted reproductive technologies	0.79 (0.47–1.33)	0.37			
Cigarette smoking during pregnancy	1.62 (0.95–2.77)	0.08			
Alcohol intake during pregnancy	0.97 (0.6–1.55)	0.89			
Drugs consumption during pregnancy	1.3 (0.4–4.24)	0.66			
Yoga or Pilates during pregnancy	0.53 (0.3–0.92)	0.03	0.92 (0.46–1.84)	0.82	−0.079
Constant			−1.976		
Data are expressed as n (%)					

STAI: State-Trait Anxiety Inventory; OR: Odds Ratio; CI: confidence interval; BMI: body-mass index. [a] Low socioeconomic status: low (never worked or unemployed >2 years). [b] Psychiatric disorders: requiring therapy for psychiatric disorder.

Table 6. Univariate and multivariate analysis of factors associated with poor maternal WHO-5 questionnaire.

Characteristics	Univariate Analysis		Multivariate Analysis		
	OR (95% CI)	*p* Value	OR (95% CI)	*p* Value	Beta Coefficient
Maternal age < 40 years	1.64 (0.95–2.84)	0.07			
Ethnicity					
White	0.76 (0.46–1.25)	0.28			
Non-white	1.31 (0.80–2.15)	0.28			
Low socioeconomic status [(a)]	1.51 (0.62–3.64)	0.36			
Study class					
Primary or secondary	1.86 (1.23–282)	0.01	1.74 (1.10–2.74)	0.02	0.553
University	0.54 (0.35–0.82)	0.01			
Medical history					
Obesity (BMI $\geq$ 30)	2.01 (1.07–3.79)	0.03	1.71 (0.88–3.32)	0.11	0.536
Diabetes mellitus	2.13 (0.72–6.26)	0.17			
Autoimmune disease	1.42 (0.81–2.50)	0.22			
Thyroid disorders	1.29 (0.61–2.72)	0.49			
Psychiatric disorders [(b)]	3.27 (1.24–8.67)	0.02	2.96 (1.07–8.25)	0.04	1.087
Chronic hypertension	2.29 (0.89–5.95)	0.09			
Chronic kidney disease	0.39 (0.05–3.21)	0.38			
Obstetric history					
Nulliparous	1.49 (0.99–2.23)	0.05	1.26 (0.81–1.94)	0.29	0.231
Previous preeclampsia	1.54 (0.56–4.24)	0.41			
Previous stillbirth	0.69 (0.08–6.23)	0.74			
Assisted reproductive technologies	0.95 (0.58–1.54)	0.82			
Cigarette smoking during pregnancy	1.55 (0.92–2.62)	0.10			
Alcohol intake during pregnancy	1.24 (0.79–1.93)	0.34			
Drug consumption during pregnancy	1.56 (0.51–4.74)	0.43			
Yoga or Pilates during pregnancy	0.54 (0.32–0.91)	0.02	0.66 (0.37–1.17)	0.16	−0.413
Constant			−1.339		
Data are expressed as n (%)					

WHO-5: World Health Organization Well-Being Index Questionnaire; OR: Odds Ratio; CI: confidence interval; BMI: body-mass index [a] Low socioeconomic status: low (never worked or unemployed >2 years). [b] Psychiatric disorders: requiring therapy for psychiatric disorder.

3.5. Maternal Sleep Quality

Poor maternal sleep quality affected 309 women (81.1%) at 34–36 weeks of gestation. While non-white ethnicity (OR 2.74; 95% CI 1.13–6.61, $p = 0.03$) and obesity (OR 2.01; 95% CI 1.07–3.79, $p = 0.03$) were significant contributors to low maternal sleep quality in the univariate analysis, in the multivariate analysis no significant contributing factors were found, as reported in Table 7.

Table 7. Univariate and multivariate analysis of factors associated with poor maternal Pittsburg questionnaire.

Characteristics	Univariate Analysis		Multivariate Analysis		
	OR (95% CI)	*p* Value	OR (95% CI)	*p* Value	Beta Coefficient
Maternal age < 40 years	1.08 (0.56–2.09)	0.81			
Ethnicity					
White	0.36 (0.15–0.88)	0.03			
Non-white	2.74 (1.13–6.61)	0.03	2.13 (0.86–5.30)	0.10	0.758
Low socioeconomic status [a]	1.18 (0.33–4.2)	0.80			
Study class					
Primary or Secondary	1.96 (1.04–3.69)	0.04	1.91 (0.98–3.77)	0.06	0.649
University	0.51 (0.27–0.96)	0.04			
Medical history					
Obesity (BMI ≥ 30)	3.1 (0.73–13.6)	0.13			
Diabetes mellitus	0.93 (0.19–4.48)	0.93			
Autoimmune disease	1.42 (0.61–3.31)	0.42			
Thyroid disorders	0.64 (0.24–1.69)	0.37			
Psychiatric disorders [b]	2.87 (0.37–22.43)	0.32			
Chronic hypertension	2.37 (0.3–18.9)	0.41			
Chronic kidney disease	0.93 (0.1–8.46)	0.95			
Obstetric history					
Nulliparous	1.16 (0.68–2)	0.59			
Use of assisted reproductive technologies	0.95 (0.51–1.8)	0.88			
Cigarette smoking during pregnancy	0.49 (0.25–0.95)	0.03	0.51 (0.25–1.02)	0.06	−0.676
Alcohol intake during pregnancy	0.76 (0.43–1.32)	0.33			
Drug consumption during pregnancy	1.94 (0.24–15.75)	0.54			
Yoga or Pilates during pregnancy	0.88 (0.48–1.58)	0.65			
Constant			1.183		

Data are expressed as n (%)

OR: Odds Ratio; CI: confidence interval; BMI: body-mass index. [a] Low socioeconomic status: low (never worked or unemployed >2 years). [b] Psychiatric disorders: requiring therapy for psychiatric disorder.

4. Discussion

Our study reveals the potential importance of assessing antenatal negative affective states in a pregnant population. Stress, anxiety, compromised well-being, and sleep disorders have been reported by a significant number of pregnant participants in our cohort. There is a possible underassessment of these conditions by obstetric-care providers in daily clinical practice and our results stress the importance of actively evaluating signs and symptoms of negative affective states and sleep quality throughout gestation.

Perceived stress and STAI-T did not change throughout pregnancy; however, STAI-S increased in the third trimester of pregnancy. Previously published studies have shown that anxiety and depressive symptoms are not homogeneous during the perinatal period [32,43,44]. Thus, nearly one quarter of participants scored as high stress and anxiety in the third trimester of pregnancy. Such percentages of perceived stress and anxiety highlight the importance of targeting these patients with clinically validated questionnaires in routine pregnancy follow-ups, with the aim of offering support interventions to these patients. Moreover, previous evidence has suggested that pregnancy-related anxiety constitutes a different concept from general anxiety. This fact could be a possible explanation for a limited measurement and assessment of anxiety in pregnancies and could also encourage the need for research in pregnancy-adapted measurement tools [45].

To the best of our knowledge, there are no data regarding the prevalence of compromised well-being in the pregnant population with which to compare our results. However, in a study conducted by Sattler et al. in a group of overweight and obese women in Europe, a prevalence of low well-being of 27% before 20 weeks of pregnancy is reported [46]. Similarly, during the COVID-19 pandemic Mortazavi et al. reported a prevalence of compromised wellbeing of 24.4% pregnant women during gestation [4]. Around 26% of our population had compromised well-being, which is a similar percentage. The WHO-5 questionnaire is considered a good screening questionnaire with high sensitivity and specificity

for clinical depression [46]. It has the advantage of being a relatively easy and quick instrument to use in daily clinical practice allowing a first detection of women with a negative affective state who could benefit from a further mental health assessment.

The prevalence of sleep disturbances in our cohort was very high: more than 80% of participants were found to have compromised sleep at 34–36 weeks of gestation. Our prevalence results are higher than expected according to the literature, ranging from 17% to 76% [29]. As suggested in previous studies, this fact could highlight the possibility that the validated cut-off for sleep questionnaires in the general population may not be valid in pregnancies, the latter requiring a higher score [30]. Moreover, we found that the results of sleep quality questionnaires worsened in the third-trimester assessment as compared to the results found in the previous weeks of gestation. The worsening of sleep quality throughout gestation identified in our cohort is in line with previous evidence: according to a meta-analysis of 24 studies, it was found that sleep disturbances tend to increase during pregnancy and clinicians should be aware that complaints of very poor sleep could require intervention [30].

Diagnosis and screening of maternal mental health have long been recommended by scientific societies. For instance, the American College of Obstetricians and Gynecologists recommends the use of a validated and standardized tool to screen pregnant women at least once during the perinatal period for symptoms of depression and anxiety [47]. However, the use of multiple questionnaires to assess maternal mental health and sleep quality can be challenging in daily clinical practice, especially in an environment with a high healthcare workload. Therefore, we believe that understanding the associated risk factors may help to target those patients at higher risk and thus facilitate daily clinical practice as they can be identified at the beginning of pregnancy. Various risk factors for antenatal negative mood states have been postulated in the previous literature [29,31–34].

In our cohort, we found that a main risk factor for maternal perceived stress, a higher level of state anxiety, and poorer well-being in the third trimester was non-university studies. In line with these results, some previous research in the pregnant population had already postulated a low educational profile as a risk factor for antenatal depression [31,32]. However, in contrast to our findings, Lancaster et al. described only a small association of lower educational levels with depressive symptoms in a systematic review [33]. In general, among the non-pregnant population, a low educational level has also been associated with anxiety and depression [48]. Our results could be explained by the fact that, as previously suggested in the literature, normally, education is likely to result in good mental health rather than come from good mental health and, in turn, education may also provide success in pursuing personal ends that include emotional well-being [48,49].

For the STAI-T personality questionnaire, we found preeclampsia in a previous pregnancy to be a potential risk factor. In a systematic review, Grigoriadis et al. found that prenatal maternal anxiety was not significantly associated with preeclampsia, although there was a significant heterogeneity across studies [50]. However, we did not find any data regarding the association between previous preeclampsia and compromised mental health in subsequent pregnancies in the previous literature. A prior history of adverse obstetric events has already been related to the symptoms of anxiety and depression [31,32,51], which could be in line with our results regarding the occurrence of preeclampsia in a previous pregnancy.

Perceived stress was also influenced in our cohort by non-white ethnicity and a maternal age of <40 years, and the latter was also found to be a risk factor for a higher score in trait anxiety among our participants. The literature also provides inconsistent findings as far as maternal age and ethnicity are concerned, as reported in the systematic reviews by Lancaster et al. [33] and Biaggi et al. [32]. In their review, Biaggi et al. described 13 studies where young age was posited as a risk factor, in contrast with 10 studies where advanced maternal age was described as a risk factor for antenatal depression and anxiety [32].

A higher level of anxiety in the third trimester and poorer maternal well-being in the third-trimester assessment were provided by the presence of a previous psychiatric

disorder. These results are in line with previously published evidence, as previous mental health disorders have been strongly related to higher anxiety in the past, in particular a history of anxiety and depression and a history of psychiatric treatment [32]. Lancaster et al. also reported an association between a personal history of depression and an increased risk of antepartum depressive symptoms [33]. Multiple studies conducted during the COVID-19 pandemic on maternal mental status proposed the presence of a previous psychiatric disorder as a risk factor for negative maternal affective states [52–55].

As for poor maternal sleep quality, no significant contributing factors were found. These findings are in contrast with those found in previous research where some risk factors could be postulated as contributors to sleep disturbances during pregnancy, such as a history of stillbirth, general health-related quality of life, or insufficient physical activity [29]. Christian et al. found that African-Americans' ethnicity and multiparity were related to poor sleep during pregnancy [56]. Other studies reported gestational age [30] or previous maternal BMI to be contributing factors [57]. Our univariate analysis also suggested ethnicity and obesity to be contributing factors; however, we could not demonstrate it in the multivariate analysis.

Finally, previous research has a well-documented association between anxiety, life stress, sleep quality, and maternal mental well-being [29,32,33]. Our results are in line with previous evidence as we found a correlation between anxiety, stress, and poorer mental well-being. In contrast, we found a low correlation between sleep quality and stress, anxiety, and mental well-being.

On the other hand, despite these associations, we believe the use of four validated questionnaires assessing different dimensions of maternal mental health may provide a more integrative approach to overall mental health, as the absence of problems in one dimension does not necessarily guarantee the same results in other aspects of mental health.

The strengths of this study were the use of various validated questionnaires with potential clinical applicability to assess different aspects of mental health: mental stress, anxiety, well-being, and sleep quality; and that they were assessed in the second and third trimester of pregnancy, which allowed an analysis of the experimented changes throughout pregnancy.

Among the study's limitations is the fact that our population was a high socioeconomic cohort, with a high education profile, and most of the participants were between 30 and 40 years of age, with a low level of ethnical variety and a low proportion of obesity and gestational diabetes. This might explain some of the findings, especially in sleep disturbances, where we could not demonstrate the contribution of these factors in multivariate analysis.

We have no data regarding the first trimester of pregnancy nor the influence that these negative affective states had on perinatal results. Moreover, the neurocognitive function was not assessed, despite its potential influence on mental health [58]. In interpreting the results, it is important to understand that the use of self-reporting instruments may potentially overestimate prevalence, but it is also important to state that they also have high clinical applicability in public health and daily obstetric-care practice. Our study confirms the importance of promoting good mental health [59], especially during pregnancy.

5. Conclusions

Maternal stress and anxiety compromised maternal well-being, and sleep quality disturbances are very frequent and not static throughout pregnancy. Screening for these conditions at different stages of pregnancy should be recommended to professionals providing obstetric care. However, in high-pressure healthcare conditions, universal screening could be challenging; therefore, knowing the risk factors associated with these conditions can help clinicians identify pregnant women at potential risk.

Author Contributions: F.C. (Francesca Crovetto), F.C. (Fàtima Crispi), and E.G. conceived and designed the study; F.C. (Francesca Crovetto) and F.C. (Fàtima Crispi) were responsible for the study protocol and ensured the correct execution of the study; F.C. (Francesca Crovetto) and F.C. (Fàtima Crispi) were the supervisors for the day-to-day running of the study, including participant

recruitment and data collection; R.P., M.G., M.L., A.N., L.B., Y.G. and I.C. were responsible for medical file revision and data collection; I.C. and L.Y. performed the data analyses; F.C. (Francesca Crovetto) supervised the data analysis; R.P., M.D.G.-R. and F.C. (Francesca Crovetto) drafted the first version of the manuscript; A.M.-A. (Anabel Martinez-Aran), I.M., T.M.O.-G., A.M.-A. (Andrés Martín-Asuero), E.V. and M.D.G.-R. contributed to the final version of the manuscript. None of the authors received any compensation for their contribution. All authors have read and agreed to the published version of the manuscript.

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STUDY 2

Impact of the COVID-19 Pandemic on Maternal Well-Being during Pregnancy.

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Impact of the COVID-19 Pandemic on Maternal Well-Being during Pregnancy

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Abstract: The outbreak of a pandemic has negative psychological effects. We aimed to determine the impact of the SARS-CoV-2 pandemic during pregnancy and identify the risk factors for maternal well-being. A multicenter, prospective, population-based study was carried out that included women (n = 1320) who were pregnant during the SARS-CoV-2 pandemic in Barcelona (Spain) compared against a pre-pandemic cohort (n = 345). Maternal well-being was assessed using the validated World Health Organization Well-Being Index Questionnaire (WHO-5 Index). Pregnant women attended during the COVID-19 pandemic showed worst WHO-5 well-being scores (median (IQR) of 56 (36–72) for the pandemic cohort vs. 64 (52–76) for the pre-pandemic cohort $p < 0.001$), with 42.8% of women presenting a poor well-being score vs. 28% for the pre-pandemic cohort ($p < 0.001$). Presence of a previous psychiatric disorder (OR 7.1; 95% CI 2.6–19, $p < 0.001$), being in the third trimester of pregnancy (OR 1.7; 95% CI 1.5–2, $p < 0.001$), or requiring hospital admission for COVID-19 (OR 4.7; 95% CI 1.4–16.7, $p = 0.014$), significantly contributed to low maternal well-being during the COVID-19 pandemic (multivariate analysis). Being infected by SARS-CoV-2 was not associated with a lower well-being score. We conclude that, during the COVID-19 pandemic, there were higher rates of poor maternal well-being; the infection of SARS-CoV-2 itself did not worsen maternal well-being, but other factors as psychiatric disorders, being in the third trimester of pregnancy or hospital admission for COVID-19 disease did.

Keywords: COVID-19; SARS-CoV-2; pandemic; well-being; pregnancy; psychiatric disorders; anxiety; depression

1. Introduction

Severe acute respiratory coronavirus 2 (SARS-CoV-2) is a global challenge for health-care sectors and individuals. Since the outbreak, many countries have adopted strict measures, such as lockdowns, aimed at mitigating the spread of the disease [1]. Previous evidence has revealed the negative psychological impact, in terms of anxiety, depression,

and post-traumatic stress symptoms [2,3] associated to the outbreak of a pandemic and its consequences on the general population, particularly on people who have quarantined [3,4].

The coronavirus 19 disease (COVID-19) has been widely studied in pregnant women. Mostly, pregnant women with SARS-CoV-2 infection remain asymptomatic and the overall rate of complications has been found to be similar to that of non-infected women [5], except close to delivery in the third trimester, where the rate of complications increases [5–7]. However, this population might still be vulnerable to medical and social risks [8]. Changes in preventive health-care seeking behavior due to lockdown and healthcare policies (prenatal care and pregnancy follow-up) may increase pregnancy-related stress disorders, have a negative effect on well-being, increase the risk of post-partum depression, and exacerbate other mental health problems [4]. During the initial spread of COVID-19 in 2020, pregnant women had less prenatal visits, relatives were not allowed to attend prenatal and postnatal visits, there was uncertainty regarding fetal transmission, and strict health measures led to social isolation [9].

Many studies have assessed the negative impact of the pandemic on maternal psychological status during pregnancy [10–12], but few studies have compared this impact to a previous pre-pandemic cohort [13] based on laboratory confirmation of SARS-CoV-2 infection [14–17]. Most current published studies on maternal psychological impact of the COVID-19 pandemic focus on depressive disorders, mental stress, or anxiety, leaving maternal well-being aside. Assessing well-being may provide a better and more general picture of the impact the pandemic has on the physical and psychological status during pregnancy.

It remains unclear whether the impact on maternal well-being is related to the COVID-19 infection itself, its severity, symptomatology, or if it is secondary to pandemic lockdown and social restrictions. The aim of this study was to examine the impact of the COVID-19 pandemic and lockdown on maternal well-being during pregnancy and identify its risk factors.

2. Materials and Methods

2.1. Study Design and Participants

A multicenter, prospective, population-based study was carried out between March 2020 and May 2020 in Barcelona, Spain [5,18]. SARS-CoV-2 infection was confirmed in all participants by the presence of antibodies and/or real-time polymerase chain reaction (RT-PCR), as described elsewhere [5]. Inclusion criteria: pregnant women who attended the participating university hospitals (Hospital Clínic, Hospital Sant Joan de Déu, and Hospital de Sant Pau) for first/second trimester screening for Down's syndrome (10–16 weeks of gestation) or admitted to the hospital for obstetric causes or delivery and were able to undergo a well-being assessment. Pregnant women referred for a SARS-CoV-2 diagnosis outside the catchment area of the participating centers were excluded from the study. The study was approved by the Ethics Committee of each of the participating hospitals (HCB: HCB-2020-0434, HSJD: PIC-56-20, HSP: IIBSP-COV-2020-38). All participants signed their informed consent before being included in the study.

The pandemic cohort was compared to a previous cohort of pregnant women recruited between February 2017 and October 2019 before the COVID-19 pandemic [19] (Table A1).

2.2. Aims of the Study

The primary purpose of the study was to evaluate maternal well-being, assessed with the World Health Organization's Well-Being Index (WHO-5) [20]. The WHO-5 consists of a five-item scale that measures quality of life and psychological well-being based on patients' feelings within the last 15 days. The raw score ranges from 0 to 25, 0 representing the worst possible and 25 the best possible quality of life. Women were classified according to their well-being status as having a poor ($\leq$52) or a favorable (>52) WHO-5 score [21]. The questionnaire was self-administered at recruitment. Comparisons of well-being scores between pandemic and pre-pandemic cohorts were carried out.

The second aim of this study was to assess maternal and pregnancy variables that may act as potential risk factors for a poorer well-being status, as well as data related to SARS-CoV-2 infection, quarantine, and lockdown.

2.3. Data Collection

Baseline and socioeconomic characteristics (working status, housing characteristics, and availability of green areas during lockdown) were obtained from a structured questionnaire, and medical and obstetric histories from the medical records at recruitment.

COVID-19 symptoms were recorded at hospital admission using a structured questionnaire that included questions on risk factors and COVID-19 suggestive symptoms noticed between mid-February 2020 and the time of SARS-CoV-2 testing. Women who tested positive, completed the same questionnaire again 4–5 weeks later. Symptomatic SARS-CoV-2 infected women were defined as having at least one of the following symptoms: fever, dry cough, anosmia or ageusia, dyspnea, myalgia, diarrhea, sore throat, skin rash, or discoloration of fingers and/or toes. More details can be found in Appendices A and B.

Pregnancy, delivery, and neonatal data were obtained from electronic medical files at delivery and during the postpartum period.

2.4. Statistical Analysis

For the primary outcome, the analyses were based on WHO-5 scorings. Secondary analyses were assessed by comparing the cohort of women who were pregnant during the SARS-CoV-2 pandemic against the pre-pandemic group. Quantitative variables were assessed for normality using Shapiro–Wilk's test: normally distributed variables were compared using the t-test and expressed as mean and standard deviation (SD). Non-normally distributed variables were compared using the U-Mann–Whitney test and expressed as median and interquartile range (IQR). Qualitative variables were compared using χ^2 or Fisher's exact tests. Logistic regression analyses were performed to assess the association between maternal well-being and potential risk factors adjusted by gestational age at recruitment. A p-value < 0.05 was considered as statistically significant. The analyses were performed on SPSS v26 (New York, NY, USA).

3. Results

3.1. Characteristics of the Study Population

During the pandemic, 1320 women were recruited; 444 (33.6%) were in the first trimester (median (IQR) gestational age 10.7 weeks (9.9–12.1)) and 876 (66.4%) in the third trimester (median (IQR) gestational age 39.7 weeks (38.6–40.6)) of pregnancy. Table 1 summarizes the baseline characteristics of the population and Table 2 shows pregnancy and neonatal outcomes. Most women (n = 851, 64.5%) had a vaginal delivery; 202 (15.3%) were positive for SARS-CoV-2 at recruitment, determined by either presence of antibodies (n = 200) and/or positive RT-PCR (n = 26) (Table A3). Table A1 summarizes the characteristics of the pre-pandemic cohort.

Table 1. Pandemic cohort baseline characteristics.

Characteristics	Total Cohort (n = 1320)
Age (years)	33.3 (29.1–37)
Ethnicity	
White	858 (65%)
Latin American	297 (22.5%)
Black	23 (1.7%)
Asian	81 (6.1%)
Others	61 (4.6%)

Table 1. *Cont.*

Characteristics	Total Cohort (*n* = 1320)
Education level	
Not educated	31 (2.3%)
Primary	86 (6.5%)
Secondary	361 (27.3%)
Vocational	191 (14.5%)
University	651 (49.3%)
Working status	
Employed	930 (70.5%)
Unemployed	262 (19.8)
Housewife	113 (8.6%)
Student	15 (1.1%)
Low socio-economic status	417 (31.6%)
Tobacco use during pregnancy	127 (9.6%)
Pre-pregnancy BMI (kg/h^2)	24.1 (4.7)
Medical history	
Obesity (BMI > 30)	157 (11.9%)
Psychiatric disorders *	28 (2.1%)
Cardiac diseases	45 (3.4%)
Respiratory disorders	65 (4.9%)
Diabetes Mellitus	18 (1.4%)
Thyroid diseases	91 (6.9%)
Obstetric history	
Nulliparous	724 (54.9%)
Assisted reproductive technologies	98 (7.4%)

Data expressed as *n* (%), median (IQR), or mean (SD). BMI: Body Mass Index. * Psychiatric disorders requiring therapy during pregnancy.

Table 2. Pandemic cohort pregnancy and neonatal outcomes.

Characteristics	Total Cohort (*n* = 1320)
Preeclampsia	57 (4.3%)
Threatened/spontaneous preterm delivery	55 (4.2%)
Preterm premature rupture of the membranes	40 (3%)
Stillbirth	7 (0.5%)
Induction of labor	509 (38.6%)
Gestational age at recruitment	
In first trimester	10.7 (9.9–12.1)
In third trimester	39.7 (38.6–40.6)
Gestational age at delivery	39.2 (2.2)
Prematurity (<37 weeks)	84 (6.4%)
Mode of delivery	
Vaginal delivery	851 (64.5%)
Operative vaginal delivery	123 (9.3%)
Cesarean section	346 (26.2%)
Fetal distress	123 (9.3%)
Female gender	616 (46.7%)
Birth weight (grams)	3280 (2985–3580)
Birth weight percentile	48 (24–74)

Table 2. *Cont.*

Characteristics	Total Cohort (*n* = 1320)
Small for gestational age (<10th centile)	154 (11.7%)
Severe small for gestational age (<3rd centile)	52 (3.9%)
Large for gestational age (>90th centile)	157 (11.9%)
5-min Apgar 5 score	9.9 (0.7)
Neonatal complications	52 (3.9%)

Data expressed as *n* (%), median (IQR), or mean (SD).

3.2. Maternal Well-Being

The median (IQR) WHO-5 score in the overall pandemic cohort was 56 (36–72); the score in 565 women (42.8%) was ≤52, suggestive of poor well-being, whereas in 755 participants (57.2%) it was >52, indicating favorable well-being (Figure 1).

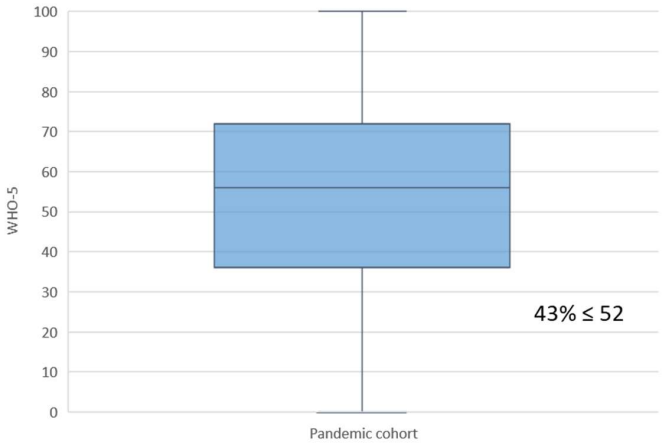


Figure 1. Maternal WHO-5 well-being outcomes for the pandemic cohort.

WHO-5 results for pregnant women during the COVID-19 pandemic (median (IQR) 56 (36–72)) were worse than for the pre-pandemic cohort (*n* = 345), (median (IQR) 64 (52–76)) ($p < 0.001$). In the pandemic cohort, 42.8% of women had a poor well-being score vs. 28% for the pre-pandemic cohort ($p < 0.001$) (Figure A1). Results were adjusted by ethnicity and psychiatric disorders (Table A1).

Table 3 shows the characteristics of the COVID-19 cohort, classified according to maternal WHO-5 well-being. No significant statistical differences were found for maternal age, ethnicity, socioeconomic status, BMI, parity, or assisted reproductive technologies. However, the existence of previous maternal psychiatric disorders was a significant contributor to low maternal well-being (4.1% vs. 0.6% in case of a favorable well-being, $p < 0.001$) (Figure 2a).

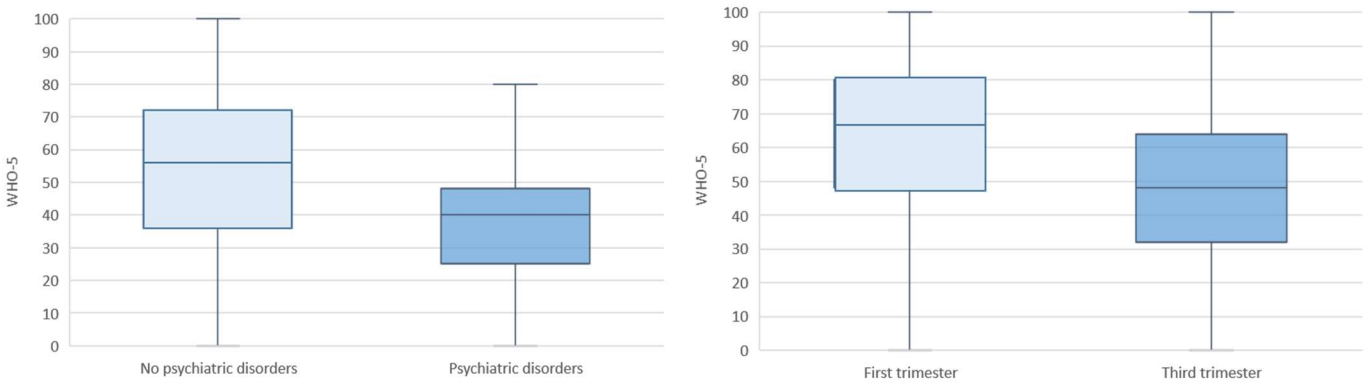
Table 3. Pandemic cohort baseline characteristics based on maternal well-being (WHO-5).

Characteristics	WHO-5 ≤ 52 (*n* = 565)	WHO-5 > 52 (*n* = 755)	*p*-Value
Age (years)	32.8 (28.8–37)	33.6 (29.6–37.2)	0.050
Ethnicity			
White	367 (65%)	491 (65%)	0.977
Latin American	135 (23.9%)	162 (21.5%)	0.294
Black	6 (1.1%)	17 (2.3%)	0.102
Asian	37 (6.5%)	44 (5.8%)	0.589
Others	20 (3.5%)	41 (5.4%)	0.105

Table 3. *Cont.*

Characteristics	WHO-5 ≤ 52 (*n* = 565)	WHO-5 > 52 (*n* = 755)	*p*-Value
Education level			
Not educated	13 (2.3%)	18 (2.4%)	0.921
Primary	35 (6.2%)	51 (6.8%)	0.683
Secondary	168 (29.7%)	192 (25.6%)	0.092
Vocational	76 (13.5%)	115 (15.2%)	0.363
University	273 (48.3%)	378 (50.1%)	0.530
Working status			
Employed	396 (70.1%)	534 (70.7%)	0.801
Unemployed	107 (19%)	154 (20.4%)	0.520
Housewife	54 (9.6%)	59 (7.8%)	0.259
Student	7 (1.2%)	8 (1.1%)	0.761
Low socio-economic status	182 (32.2%)	235 (31.1%)	0.674
Tobacco use during pregnancy	53 (9.4%)	74 (9.8%)	0.798
BMI (kg/h^2)	24 (4.6)	24.2 (4.8)	0.340
Medical history			
Obesity (BMI > 30)	67 (11.9%)	90 (11.9%)	0.972
Psychiatric disorders *	23 (4.1%)	5 (0.7%)	<0.001
Cardiac diseases	13 (2.3%)	32 (4.2%)	0.055
Respiratory disorders	29 (5.1%)	36 (4.8%)	0.762
Diabetes Mellitus	6 (1.1%)	12 (1.6%)	0.414
Thyroid diseases	30 (5.3%)	61 (8.1%)	0.049
Obstetric history			
Nulliparous	314 (55.6%)	411 (54.4%)	0.681
Assisted reproductive technologies	36 (6.4%)	62 (8.2%)	0.207

Data expressed as *n* (%), median (IQR), or mean (SD). BMI: Body Mass Index. * Psychiatric disorders requiring therapy during pregnancy.



(**a**) (**b**)

Figure 2. Maternal WHO-5 well-being outcomes for the pandemic cohort based on the presence of psychiatric disorders (**a**) and trimester of pregnancy (**b**).

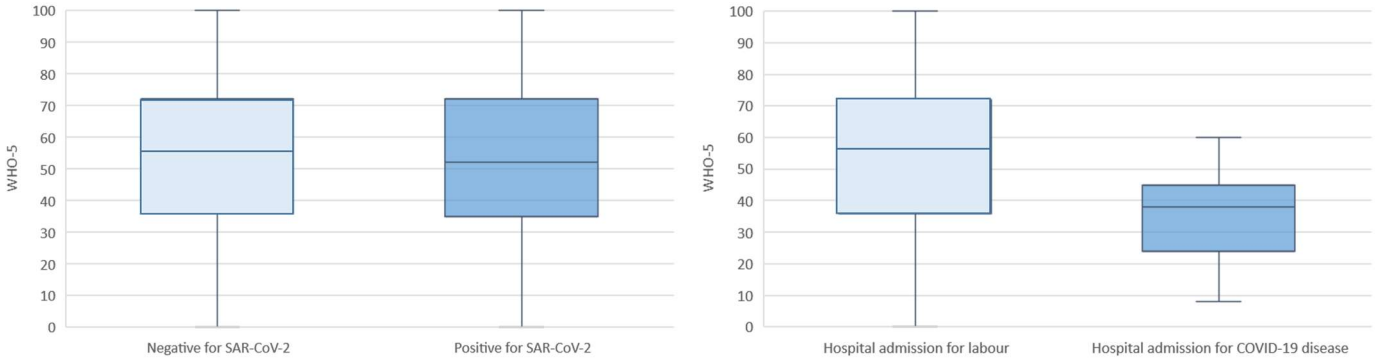
Regarding pregnancy and neonatal outcomes, being in the third trimester of pregnancy was significantly associated to worse maternal well-being (median (IQR) score 48 (I32–64) ($p < 0.001$) (Figure 2b). This association was not seen for preeclampsia, prematurity, cesarean section, or fetal distress among others (Table 4).

Table 4. Pregnancy and neonatal outcomes for the pandemic cohort based on WHO-5 well-being.

Characteristics	WHO-5 ≤ 52 (*n* = 565)	WHO-5 > 52 (*n* = 755)	*p*-Value
Trimester			<0.001
First trimester	117 (20.7%)	327 (43.3%)	
Third trimester	448 (79.3%)	428 (56.7%)	
Preeclampsia	28 (5%)	29 (3.8%)	0.324
Threatened/spontaneous preterm labor	29 (5.2%)	25 (3.6%)	0.147
Preterm premature rupture of the membranes	15 (2.7%)	25 (3.3%)	0.491
Stillbirth	3 (0.5%)	4 (0.5%)	0.998
Induction of labor	226 (40%)	283 (37.5%)	0.353
Gestational age at delivery	39.1 (2.3)	39.3 (2.1)	0.316
Prematurity (<37 weeks)	40 (7.1%)	44 (5.8%)	0.357
Mode of delivery			
Vaginal delivery	361 (63.9%)	490 (64.9%)	0.705
Operative vaginal delivery	56 (9.9%)	67 (8.9%)	0.551
Cesarean section	148 (26.2%)	198 (26.2%)	0.990
Fetal distress	61 (10.8%)	62 (8.2%)	0.110
Female gender	269 (47.6%)	347 (46%)	0.552
Birth weight (grams)	3260 (2940–3560)	3295 (3020–3595)	0.076
Birth weight percentile	45 (21–74)	50 (27–74)	0.47
Small for gestational age (<10th centile)	67 (11.9%)	87 (11.5%)	0.851
Severe small for gestational age (<3rd centile)	22 (3.9%)	30 (4%)	0.941
Large for gestational age (>90th centile)	68 (12%)	89 (11.8%)	0.891
5-min Apgar score	9.8 (0.8)	9.9 (0.7)	0.268
Neonatal complications	29 (5.1%)	23 (3%)	0.054

Data expressed as *n* (%), median (IQR), or mean (SD).

Regarding SARS-CoV-2 infection, the infection itself did not have an effect on the level of maternal well-being (p = 0.812) (Figure 3a). However, presence of severe symptoms (fever, cough, or dyspnea) and hospital admission for COVID-19 were associated with a lower well-being score (Table 5 and Figure 3b). No SARS-CoV-2 infection cases were reported in newborns.



(**a**) (**b**)

Figure 3. WHO-5 well-being outcomes for the pandemic cohort based on SARS-CoV-2 infectious status: (**a**) positive vs. negative women; (**b**) women hospitalized for moderate-severe COVID-19.

Table 5. Symptoms and diagnosis of SARS-CoV-2 infection and COVID-19 disease in the pandemic cohort based on the level of maternal WHO-5 well-being.

Characteristics	WHO-5 ≤ 52 (*n* = 565)	WHO-5 > 52 (*n* = 755)	*p*-Value
Positive SARS-CoV-2 testing	88 (15.6%)	114 (15.1%)	0.812
Symptoms of SARS-CoV-2 infection within the last 10 weeks	95 (16.8%)	87 (11.5%)	0.006
Fever	25 (4.4%)	19 (2.5%)	0.056
Dry cough	44 (7.8%)	31 (4.1%)	0.004
Difficulty breathing or shortness of breath	17 (3%)	12 (1.6%)	0.082
Diarrhea	20 (3.5%)	16 (2.1%)	0.117
Other respiratory symptoms	9 (1.6%)	8 (1.2%)	0.534
Myalgia	17 (3%)	17 (2.3%)	0.390
Skin rash	5 (0.9%)	4 (0.5%)	0.438
Loss of taste or smell	15 (2.7%)	12 (1.6%)	0.176
Other	10 (1.8%)	16 (2.1%)	0.651
Combination of symptoms predictable for SARS-CoV-2 infection			
At least two symptoms or anosmia	44 (7.8%)	39 (5.2%)	0.052
At least three symptoms or anosmia	22 (3.9%)	20 (2.6%)	0.202
Fever, cough and dyspnea	8 (1.4%)	1 (0.1%)	0.005
Symptom-relatedCOVID-19 severity			
Mild	2 (14.5%)	79 (10.5%)	0.026
Moderate	5 (0.9%)	7 (0.9%)	0.936
Severe	8 (1.4%)	1 (0.1%)	0.005
COVID-19 disease			
Hospital admission for COVID-19 disease	15 (2.7%)	3 (0.4%)	<0.001
Pneumonia	3 (0.5%)	1 (0.1%)	0.192
Severe pneumonia	2 (0.4%)	1 (0.1%)	0.403
Oxygen support	2 (0.4%)	1 (0.1%)	0.403
Admission to intensive care unit	1 (0.2%)	1 (0.1%)	0.837
Invasive ventilatory support	1 (0.2%)	0 (0%)	0.248

Data are expressed as *n* (%). RT-PCR: Real Time Polymerase chain reaction; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

Multivariate analyses revealed significant contribution to low maternal well-being with the presence of psychiatric disorders (OR 7.1; 95% CI 2.6–19, $p < 0.001$), being in the third trimester of pregnancy (OR 1.7; 95% CI 1.5–2, $p < 0.001$), or hospital admission for COVID-19 (OR 4.7; 95% CI 1.4–16.7, $p = 0.014$) (Table 6). No association was found between SARS-CoV-2 infection itself and a reduced well-being score.

Table 6. Multivariate analysis of factors associated to poor maternal WHO-5 well-being in the pandemic cohort.

	Univariate Analysis		Multivariate Analysis		
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-Value	Betta Coefficient
Baseline maternal characteristics					
Age (years)	0.98 (0.96–1)	0.051			
Gestational age at recruitment (weeks)	1.04 (1.03–1.05)	<0.001			
Non-European ethnicity	1 (0.8–1.3)	0.977			
Low socio-economic status	1 (0.8–1.3)	0.674			
Tobacco use during pregnancy	0.95 (0.7–1.4)	0.789			
Psychiatric disorders	6.4 (2.4–16.9)	<0.001	7.1 (2.6–19)	<0.001	1.947
Thyroid diseases	0.6 (0.4–1)	0.051			
Nulliparity	1 (0.8–1.3)	0.681			
Assisted reproductive techniques	0.7 (0.5–1.2)	0.208			
Pregnancy outcomes					
Trimester (first vs. third)	1.7 (1.5–1.9)	<0.001	1.7 (1.5–2)	<0.001	0.537
Induction of labor	1.1 (0.9–1.4)	0.353			
Cesarean section	0.99 (0.8–1.3)	0.99			
SARS-CoV-2 status					
Positive SARS-CoV-2 testing	1 (0.8–1.4)	0.812			
Presence of at least one COVID-19 symptom	1.5 (1.1–2.1)	0.006			
Presence of fever, cough and dyspnea	10.8 (1.3–86.8)	0.025			
Presence of severe COVID-19 symptoms	10.8 (1.3–86.8)	0.025			
Hospital admission for COVID-19	6.8 (1.9–23.7)	0.002	4.8 (1.4–16.7)	0.014	1.565
Constant					−1.606

Data are expressed as *n* (%). OR: Odds Ratio; CI: confidence interval; SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2; COVID-19: Coronavirus 19 disease.

3.3. Lockdown Characteristics

Four hundred and eighty participants of the pandemic cohort answered a structured questionnaire on lockdown characteristics (Table A2). Most pregnant women remained isolated in their usual residence (n = 448; 93.3%) without older people at home (n = 434; 90.4%) and the majority (n = 439; 91.5%) were not concerned with the general impact of the pandemic, although 332 (69.2%) communicated they were worried about their pregnancy and their fetus. No significant contributors to maternal well-being status were identified (Table A2).

4. Discussion

4.1. Main Findings

The well-being score in almost half (43%) of our study population is low. This has been related to symptoms of depression [21]. Thus, maternal well-being status during the COVID-19 pandemic is affected. This is more evident when we compare pandemic versus pre-pandemic cohorts, where 28% of the latter cohort had poor well-being scores. Additionally, there are risk factors that contribute to a worse well-being during pregnancy, such as previous psychiatric disease, being in the third trimester of pregnancy, and hospital admission for COVID-19 disease. The infection of SARS-CoV-2 itself did not increase the risk of a lower well-being condition, but the severity of COVID-19 disease requiring hospitalization did.

Well-being is broadly defined as 'the quality and state of a person's life' [22] and consists of two components: feeling healthy and relatively robust and being able to carry out ones job and other tasks satisfactorily [23]. Fear related to childbirth is multidimensional and, under normal circumstances, only around 20% of pregnant women experience excessive concern regarding future events in pregnancy [23]. Feelings of well-being are key to the overall health of an individual but can be affected by physical and emotional trauma.

Several studies have reported a compromised maternal mental status during the COVID-19 pandemic [3,12,16,24]. Higher depressive rates in comparison to pre-pandemic subjects [13] and prevalence of depressive and anxiety symptoms ranging around 15–19% and 11–31%, respectively, [12,16] have been found. However, most of these works are based on maternal depression and anxiety scales and a limited number use maternal well-being as an assessment of maternal physical, mental, and social health [23].

Few studies have compared pandemic cohort data to a previous pre-pandemic cohort, suggesting worse maternal anxiety and depression levels in patients assessed during the COVID-19 pandemic. Wu et al. reported higher depression symptoms in patients during the pandemic in comparison to a pre-pandemic cohort and found a positive association with the number of newly COVID-19 confirmed cases, suspected cases, and deaths [13]. Similarly, in a study by Berthelot et al., the authors found that COVID-19 pandemic-affected women were more likely to present depressive and anxiety symptoms, especially those with a previous psychiatric diagnosis or low income [25]. Zanardo et al. reported higher scores for anhedonia and depression in comparison to 100 previous patients [26]. Interestingly, Dong et al. found that anxiety levels of pregnant women were the same as before the pandemic, while the level of depression was significantly higher. The authors reported no differences in terms of gestational age or testing positive for Sars-CoV-2 infection [17]. Perzow et al. compared 135 patients pre- and post-pandemic and determined higher levels of anxiety and depression during the pandemic [27]. To the best of our knowledge, ours is the first study that assesses maternal well-being before and after the pandemic.

Our results suggest that the existence of a previous psychiatric maternal condition is as a risk factor for worse maternal well-being. Similarly, some studies have reported that a previous psychiatric disorder diagnosed in pregnant women is as a risk factor for depression symptoms during the COVID-19 pandemic [25,28,29]. The stage of pregnancy had a unique association with anxiety and the level of well-being. Zeng et al. reported that the third trimester of pregnancy at the time of the COVID-19 pandemic seemed to be associated with a worse maternal well-being, with even worse results in comparison to the

post-partum period [12]. On the contrary, Saccone et al. found worse results in anxiety and psychological impact in pregnant women in the first trimester [24]. Other authors found no differences according to gestational age [11,17,30].

COVID-19 symptoms and infection have been described as anxiety factors [31] and predictors for post-traumatic stress disorder [32]. However, these studies did not consider the differences between confirmed SARS-CoV-2 infected and healthy patients. SARS-CoV-2 infection may increase the level of anxiety and worsen mental condition; our data do not confirm this hypothesis as found in other studies with smaller sample sizes [15,17]. We report worse maternal well-being in SARS-CoV-2 infected mothers with severe symptoms or requiring hospital admission due to COVID-19 disease for respiratory and or medical support according to our center protocols at the time of the study.

4.2. Clinical Relevance

Our results suggest the potential utility of maternal well-being screening during the COVID-19 pandemic, especially in patients with a previous diagnosis of mental illness and in their third trimester of pregnancy, close to delivery. There is no negative effect of SARS-CoV-2 maternal infection on their well-being. However, well-being is affected in pregnant women who require hospital admission for moderate to severe COVID-19 disease, who might benefit from a psychological support during their hospital stay.

4.3. Strengths and Limitations

Some of the strengths of this study include a very well characterized population of pregnant women, laboratory confirmation of SARS-CoV-2 infection in all women in different pregnancy stages and during the first wave of COVID-19 pandemic, where strict restriction measures were applied. The short and simple WHO-5 questionnaire can screen depressive symptoms and evaluate subjective well-being in pregnant populations, which can be helpful in daily clinical practice, especially when healthcare pressure is high. There are several limitations to this study. The WHO-5 questionnaire was self-administration with no psychiatric screening thereafter, there were no postpartum depression or anxiety symptoms follow-ups, and baseline characteristics of the pre-pandemic and pandemic cohorts were not identical. To overcome these limitations, we applied careful statistical adjustments. Moreover, our study did not include a follow-up of postpartum depression or anxiety symptoms that could be considered in future studies.

5. Conclusions

In conclusion, the COVID-19 pandemic is a challenge for pregnant women in terms of well-being, especially in their third trimester of pregnancy. Previous psychiatric disorders are associated to higher risk of poor well-being. The well-being of pregnant women testing positive for SARS-CoV-2 infection is not affected, except when presenting severe infection-related symptomatology or requiring hospitalization due to COVID-19 disease, in which cases poorer well-being was reported.

Author Contributions: F.C. (Francesca Crovetto), F.C. (Fàtima Crispi) and E.G. conceived and designed the study. E.L., F.C. (Francesca Crovetto) and M.D.G.-R. were responsible of the study protocol at each hospital and ensured the correct execution of the study. F.C. (Francesca Crovetto), F.C. (Fàtima Crispi) and E.L. were the supervisors at each of the three hospitals for day-to-day running of the study, including participant recruitment and data collection. R.P., M.L., C.T., A.C., D.B. and I.C. were responsible of medical file revision and data collection at the three participating hospitals. I.C., J.S., M.F. and L.Y. performed the data analyses. F.C. (Francesca Crovetto) supervised the data analysis. R.P. and F.C. (Francesca Crovetto) drafted the first version of manuscript. E.G. is the principal investigator of the project. None of the authors received any compensation for their contribution. All authors have read and agreed to the published version of the manuscript.

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Appendix A.

Appendix A.1. COVID-19 Evaluation

COVID-19 symptoms were recorded at hospital admission using a structured self-prepared questionnaire that included questions about risk factors and COVID-19 suggestive symptoms noticed between mid-February 2020 and the time of SARS-CoV-2 testing. All positive women completed the same questionnaire again 4–5 weeks later. Symptomatic SARS-CoV-2 infected women were defined as having at least one of the following symptoms: fever, dry cough, anosmia or ageusia, dyspnea, myalgia, diarrhea, sore throat, skin rash, or discoloration of fingers and/or toes.

Appendix A.2. Sample Collection

Maternal blood samples were drawn from peripheral veins in first and third trimester participants, at recruitment. Samples were centrifuged at 1500× *g* for 10 min at 4 °C and sera immediately stored at −80 °C until further analysis. For SARS-CoV-2 IgG and IgM/IgA antibody determination, the COVID-19 VIRCLIA® Monotest (Vircell Microbiologist, Granada, Spain) was used. Indeterminate results were re-tested (VITROS® Immunodiagnostic Products Anti-SARS-CoV2 Total Tests, Ortho Clinical Diagnostics, Rochester, NY, USA) and classified as positive or negative. Likewise, results positive for IgM + IgA but negative for IgG in women reporting no symptoms suggestive of COVID-19 during the 10 weeks prior testing were re-tested with Luminex and classified as positive or negative [33]. A serological result was considered positive if any of the following were found: (a) IgG positive, (b) IgM + IgA positive in women with symptomatic COVID-19, (c) IgM + IgA positive confirmed by two tests (Vircell and Luminex).

Nasopharyngeal swab samples for SARS-CoV-2 RNA RT-PCR were collected in all third trimester pregnancies recruited at hospital admittance. Samples were collected in Micronics tubes with Zymo DNA/RNA Shield Lysis buffer. RNA was extracted using the Quick-DNA/RNA Viral MagBead kit (Zymo) and the TECAN Dreamprep robot. Five microliters of RNA solution were added to 15 µL of the rRT-PCR master mix (Luna Universal Probe One-Step RT-qPCR Kit; New England Biolabs) and used for amplification of the SARS-CoV-2 N1 and N2 regions, as well as the human RNase P gene as control, as described in the CDC-006-00019 CDC/DDID/NCIRD/Division of Viral Diseases protocol released 3/30/2020. A SARS-CoV-2 positive result was considered if Ct values for N1, N2, and RNase P were below 40. Samples discordant for N1 and N2 were repeated and samples with a Ct $\geq$ 40 for RNase P were considered as invalid.

SARS-CoV-2 infection was defined by either a positive serological result or RT-PCR in nasopharyngeal swabs.

Appendix B.

Table A1. Baseline characteristics of pre-pandemic and COVID-19 pandemic pregnant women cohorts.

Characteristics	Pre-Pandemic (n = 345)	Pandemic (n = 1320)	p-Value
Ethnicity			
White	279 (80.9%)	858 (65%)	<0.001
Latin American	49 (14.2%)	297 (22.5%)	0.001
Black	6 (1.7%)	23 (1.7%)	0.997
Asian	6 (1.7%)	81 (6.1%)	0.001
Others	5 (1.4%)	61 (4.6%)	0.007
Tobacco use during pregnancy	27 (7.8%)	127 (9.6%)	0.305
Pre-pregnancy BMI (kg/h^2)	23.8 (4.8)	24.1 (4.7)	0.29
Medical history			
Obesity (BMI > 30)	39 (11.3%)	157 (11.9%)	0.762
Psychiatric disorders *	15 (4.3%)	28 (2.1%)	0.020
Thyroid diseases	31 (9%)	91 (6.9%)	0.184
Obstetric history			
Nulliparous	203 (58.8%)	725 (54.9%)	0.192

Data are expressed as n (%) or median (IQR) or mean (SD). BMI: Body mass index. * Psychiatric disorders requiring therapy during pregnancy.

Table A2. Self-administered questionnaire on COVID-19 pandemic-related conditions.

Characteristics	Total Cohort (n = 480)	WHO-5 $\leq$ 52	WHO-5 >52	p-Value
SARS-CoV-2 diagnosis by laboratory test				0.079
Yes	7 (1.5%)	10 (3.4%)	2 (1%)	
No	473 (98.5%)	287 (96.6%)	207 (99%)	
Contact with a symptomatic SARS-CoV-2 person				0.098
Yes	42 (8.8%)	21 (7%)	24 (11.2%)	
No	438 (91.3%)	278 (93%)	190 (88.8%)	
Know someone diagnosed by SARS-CoV-2				0.247
Yes	129 (26.9%)	74 (24.4%)	62 (29%)	
No	351 (73.1%)	229 (75.6%)	152 (71%)	
Degree of concern about SARS-CoV-2 epidemic				0.088
I'm very worried	192 (40%)	112 (37.2%)	94 (44.1%)	
I'm quite worried	222 (46.3%)	141 (46.8%)	97 (45.5%)	
I'm a little worried	59 (12.3%)	45 (15%)	18 (8.5%)	
Don't care	7 (1.5%)	3 (1%)	4 (1.9%)	
Worry of getting the disease yourself or a family member				0.537
I'm very worried	279 (58.1%)	170 (56.1%)	133 (62.1%)	
I'm quite worried	159 (33.1%)	107 (35.3%)	63 (29.4%)	
I'm a little worried	40 (8.3%)	25 (8.3%)	17 (7.9%)	
Don't care	2 (0.4%)	1 (0.3%)	1 (0.5%)	
Effect on the pregnancy and fetus concerns				0.220
I'm very worried	332 (69.2%)	202 (66.9%)	156 (72.9%)	
I'm quite worried	84 (17.5%)	58 (19.2%)	32 (15%)	
I'm a little worried	53 (11%)	33 (10.9%)	24 (11.2%)	
Don't care	11 (2.3%)	9 (3%)	2 (0.9%)	
Personal economic concern				0.944
I'm very worried	226 (47.1%)	146 (48.2%)	102 (47.7%)	
I'm quite worried	148 (30.8%)	88 (29%)	66 (30.8%)	
I'm a little worried	86 (17.9%)	55 (18.2%)	38 (17.8%)	
Don't care	20 (4.2%)	14 (4.6%)	8 (3.7%)	
Impact on global economy concerns				0.110
I'm very worried	199 (41.5%)	124 (40.9%)	93 (43.5%)	
I'm quite worried	198 (41.3%)	116 (38.3%)	94 (43.9%)	
I'm a little worried	72 (15%)	55 (18.2%)	24 (11.2%)	
Don't care	11 (2.3%)	8 (2.6%)	3 (1.4%)	

Table A2. *Cont.*

Characteristics	Total Cohort (n = 480)	WHO-5 $\leq$ 52	WHO-5 >52	p-Value
Excessive worrying				0.092
Yes	41 (8.5%)	36 (11.9%)	14 (6.5%)	
No	439 (91.5%)	267 (88.1%)	200 (93.5%)	
Does the pregnant woman have enough information regarding the effects of the virus on pregnancy and the fetus				0.332
Yes	216 (45%)	141 (47-2%)	89 (41.6%)	
No	264 (55%)	160 (52.8%)	125 (58.4%)	
Isolation in primary residence				0.515
Yes	448 (93.3%)	277 (91.4%)	199 (93%)	
No	32 (6.7%)	26 (8.6%)	25 (7%)	
People at risk living at home				0.548
Yes	46 (9.6%)	27 (8.9%)	23 (10.8%)	
No	434 (90.4%)	275 (91%)	189 (89.2%)	
Terrace or garden at home				0.809
Yes	251 (52.3%)	158 (53.2%)	111 (52.1%)	
No	229 (47.7%)	139 (46.8%)	102 (47.9%)	
Work				0.748
No	419 (87.3%)	266 (88.1%)	184 (86%)	
Yes, from home	51 (10.6%)	29 (9.6%)	25 (11.7%)	
Yes, at my usual place of work	10 (2.1%)	7 (2.3%)	5 (2.3%)	
How many times a week does she go out				0.352
Never	162 (33.8%)	97 (32%)	78 (36.4%)	
One or two times a week	232 (48.3%)	146 (48.2%)	106 (49.5%)	
Between three and five times a week	52 (10.8%)	36 (11.9%)	19 (8.9%)	
Six or more times a week	34 (7.1%)	24 (7.9%)	11 (5.1%)	
Coping with isolation				<0.001
Very well	94 (19.6%)	69 (23.1%)	28 (13.1%)	
Pretty well	309 (64.4%)	197 (65.9%)	136 (63.8%)	
Poorly	68 (14.2%)	29 (9.7%)	42 (19.7%)	
Very poorly	9 (1.9%)	4 (1.3%)	7 (3.3%)	
Mental health before the pandemic				0.069
Excellent	106 (26.6%)	75 (29.8%)	38 (21.5%)	
Very good	180 (45.2%)	115 (45.6%)	81 (45.8%)	
Good	97 (24.4%)	56 (22.2%)	46 (26%)	
Regular	11 (2.8%)	4 (1.6%)	10 (5.6%)	
Bad	4 (1%)	2 (0.8%)	2 (1.1%)	

Data are expressed as n (%). SARS-CoV-2: severe acute respiratory syndrome coronavirus.

Table A3. Prevalence of SARS-CoV-2 infection during pregnancy.

Characteristics	Total Cohort (n = 1320)
SARS-CoV-2 positive (RT-PCR and/or Ab)	202 (15.3%)
First trimester	82 (40.6%)
Third trimester	120 (59.4%)
RT-PCRa positive	26 (3%)
Ab for SARS-CoV-2 infection IgM/A/G	
Negative	1120 (84.8%)
Positive	200 (15.2%)

Data are expressed as n (%) or median (IQR); SARS-CoV-2: severe acute respiratory syndrome coronavirus; RT-PCR: Real Time Polymerase chain reaction; Ab: Antibody. Data available only for 876 cases (Third trimester participants).

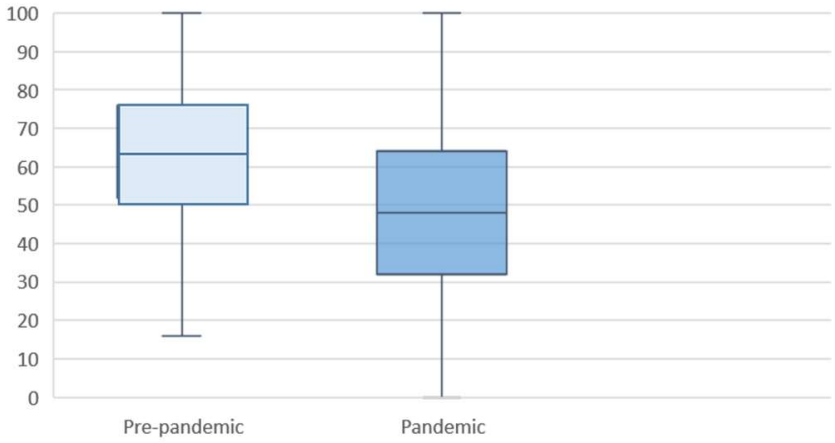

Figure A1. WHO-5 well-being level in pre-pandemic (n = 345) and pandemic (n = 1320) cohorts.

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STUDY 3

Effects of a Mediterranean Diet Intervention on Maternal Stress, Well-Being, and Sleep Quality throughout Gestation – The IMPACT-BCN Trial.

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Effects of a Mediterranean Diet Intervention on Maternal Stress, Well-Being, and Sleep Quality throughout Gestation—The IMPACT-BCN Trial

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Abstract: Stress and anxiety are frequent occurrences among pregnant women. We aimed to evaluate the effects of a Mediterranean diet intervention during pregnancy on maternal stress, well-being, and sleep quality throughout gestation. In a randomized clinical trial, 1221 high-risk pregnant women were randomly allocated into three groups at 19–23 weeks' gestation: a Mediterranean diet intervention, a Mindfulness-Based Stress Reduction program, or usual care. All women who provided self-reported life-style questionnaires to measure their anxiety (State Trait Anxiety Inventory (STAI), Perceived Stress Scale (PSS)), well-being (WHO Five Well Being Index (WHO-5)), and sleep quality (Pittsburgh sleep quality index (PSQI)) at enrollment and at the end of the intervention (34–36 weeks) were included. In a random subgroup of 106 women, the levels of cortisol and related metabolites were also measured. At the end of the intervention (34–36 weeks), participants in the Mediterranean diet group had significantly lower perceived stress and anxiety scores (PSS mean (SE) 15.9 (0.4) vs. 17.0 (0.4), $p = 0.035$; STAI-anxiety mean (SE) 13.6 (0.4) vs. 15.8 (0.5), $p = 0.004$) and better sleep quality (PSQI mean 7.0 ± 0.2 SE vs. 7.9 ± 0.2 SE, $p = 0.001$) compared to usual care. As compared to usual care, women in the Mediterranean diet group also had a more significant increase in their 24 h urinary cortisone/cortisol ratio during gestation (mean 1.7 ± SE 0.1 vs. 1.3 ± SE 0.1, $p < 0.001$). A Mediterranean diet intervention during pregnancy is associated with a significant reduction in maternal anxiety and stress, and improvements in sleep quality throughout gestation.

Keywords: Mediterranean diet; pregnancy; anxiety; well-being; sleep quality

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1. Introduction

The Mediterranean diet (MedDiet) has several positive effects on individual health: randomized trials demonstrated its contribution to improved cardiovascular profiles and reduced major cardiovascular events in individuals at risk of [1] diabetes, inflammatory-based disorders, cancer, and cognitive decline [2–4]. Additionally, there has been increasing interest of the effects of a MedDiet on mental health, stress, and quality of life in general [5]. The role of the diet, particularly the MedDiet, in the development of mental disorders, has become a recent research focus over the past decade [6]. Several studies evaluated the effect of a MedDiet intervention on the reduction in depressive symptoms and the improvement in quality of life in individuals with major depressive disorders [7,8]. In a secondary analysis of the PREvención con DIeta MEDiterránea (PREDIMED) study, a reduced risk in depression was observed in participants with type 2 diabetes allocated to the group receiving a MedDiet supplemented by nuts (hazard ratio 0.59 (95% confidence interval (CI) 0.36 to 0.98)) [9]. A recent review based on 37 studies confirmed the association between (poly)phenols consumption and the risk of depression, and a reduction in the severity of depressive symptoms [10]. Some authors hypothesized that a high-quality diet, rich in fiber, antioxidant dietary components and omega-3-polyunsaturated fatty acids, may be linked to a reduced risk of depression, anxiety, and stress [11], which could provide new potential methods for the treatment and prevention of mental disorders in general. Moreover, it has been described that a dysregulated redox signaling is a key factor in the pathophysiology of mental disorders, especially in depression, and increased reactive oxygen and nitrogen species were observed in these patients [12,13].

Stress and anxiety are frequent occurrences among pregnant women. Peripartum anxiety disorders are more prevalent than previously thought, as 1 in 5 women can suffer from them [14]. Mental disorders can appear before pregnancy, with a changing course during pregnancy and postpartum. These findings highlight the need for screenings for stress-related disorders and education by different health professionals from the early stages of pregnancy. Several studies have shown the effectiveness of non-pharmacological treatments in the improvement in stress and other mental disorders during pregnancy, such as mindfulness meditation, biofeedback, or exercise such as yoga [15]. However, there is paucity of data regarding the dietary approach to these conditions during pregnancy. Interestingly, a recent observational study revealed an association between the MedDiet and anxiety [16]. Moreover, the production of reactive oxygen and nitrogen species production, as well as individual antioxidant capacity, is influenced by several dietary factors. A dietary intervention promoting plant-based foods that are rich in antioxidants, such fruits, vegetables, extra-virgin olive oil, and whole-grain cereals, may modulate the individual antioxidant capacity, explaining the improvements in mental wellbeing [12]. Thus, randomized clinical trials are needed to establish the potential effects of dietary patterns on mental health, avoiding the confusion attributed to the co-occurrence of other lifestyle-related and sociodemographic factors.

During pregnancy, evidence has been provided regarding the potential beneficial effects that structured dietary interventions based on a MedDiet can have, not only on pregnant women [9,13,14], but also their offspring and the pregnancy itself. In a recent randomized clinical trial, pregnant individuals at high risk for small-for-gestational-age newborns (SGA) who followed a structured MedDiet intervention significantly reduced the incidence of newborns being born small (with birth weight below the 10th percentile) and other perinatal complications [17]. However, the influence of MedDiet on maternal wellbeing during pregnancy remains to be determined.

The present study aimed to evaluate the influence of a structured intervention during pregnancy based on a MedDiet on maternal stress and anxiety, mindful state, quality of life and sleep.

2. Materials and Methods

2.1. Study Design, Population and Ethics

Improving Mothers for a better PrenAtal Care Trial BarCeloNa (IMPACT BCN) was a parallel, unblinded randomized clinical trial conducted at BCNatal (Hospital Clínic and Hospital Sant Joan de Déu), a large referral center for maternal–fetal and neonatal medicine in Barcelona, Spain. Details of the trial are provided in the protocol of the study [18], approved by the Institutional Review Board (HCB-2016-0830) before any participant enrolment. All individuals who agreed to participate provided written informed consent before randomization. Participants were screened for eligibility during routine second trimester ultrasound scans (19–23.6 weeks of gestation) for being at high risk of developing an SGA newborn [19], and were randomly assigned 1:1:1, based on a computerized random number generator, to one of the three study groups: a MedDiet supplemented with extra-virgin olive oil and walnuts; a stress reduction intervention based on the Mindfulness-Based Stress Reduction (MBSR) program; or usual care without any intervention (control group). For this specific study, only women belonging to the group of MedDiet and usual care who provided lifestyle questionnaires were included. The trial was registered in ClinicalTrials.gov Identifier: NCT03166332.

2.2. Interventions and Measurements

2.2.1. Mediterranean Diet Program

The dietary intervention, adapted from the PREDIMED trial [20], aimed to change the general dietary pattern instead of focusing on changes in single foods or macronutrients. Participants were encouraged to increase their intake of whole-grain cereals ($\geq$5 servings/d); vegetables and dairy products ($\geq$3 servings/d); fresh fruit ($\geq$2 servings/d); and legumes, nuts, fish, and white meat ($\geq$3 servings/week), as well as increasing their olive oil use for cooking and dressings. To achieve a personalized goal, personal and individual recommendations were introduced to the participant's diet according to height, weight, culture, and dietary preferences. Dieticians conducted 30 min face-to-face interviews at enrollment and monthly until the end of intervention (34–36 weeks' gestation). Two weeks following each face-to-face visit, participants underwent telephone interviews. In addition, all participants received extra-virgin olive oil (2 L every month) and 15 g of walnuts per day (450 g every month) at no cost. Additional details of the intervention are provided elsewhere [18]. No intervention or advice regarding mental health, well-being, anxiety, stress, or sleep quality were provided to the participants allocated to the Mediterranean diet group.

2.2.2. Usual Care (Control Group)

Women randomized into this group received usual pregnancy care as per institutional protocols (no intervention), and lifestyle questionnaires were collected at enrollment and at the end of intervention (34–36 week's gestation). No intervention or advice regarding mental health, well-being, anxiety, stress, or sleep quality were provided to the participants allocated to the control group.

2.3. Outcomes

In this trial sub-analysis, the main aim was to investigate the influence of a Mediterranean diet intervention program during pregnancy on maternal stress, anxiety, well-being, mindful state, and sleep quality. Additionally, in a randomly selected subgroup of participants, the levels of cortisol, cortisone and other intermediate related metabolites were measured at the beginning and at the end of the intervention in 24 h urine samples.

2.4. Data Collection

The data of participants included in the study were anonymized and entered in an electronic case report form. Investigators collected maternal sociodemographic and clinical data.

All individuals included in the trial had a baseline visit (19–23 weeks of gestation) and a final visit (34–36 weeks of gestation) with a trained dietitian to assess their diet using a validated 151-item food-frequency questionnaire [21], 7-day dietary registry and the 17-item MedDiet adapted to pregnancy adherence score (score range: 0–17). All participants also provided self-report lifestyle questionnaires to measure their anxiety and stress (State-trait Anxiety Inventory (STAI) Anxiety and Personality [22], range 0–80); Perceived Stress Scale (PSS) [23], range 0–40; well-being (WHO Five Well Being Index (WHO-5) [24], range 0–100); mindful state (WHO Five Facet Mindfulness Questionnaire (FFMQ) [25], range 8–40 for the observation, description, awareness, and nonjudgmental facets, respectively, and range 7–35 for nonreactivity facet); sleep quality (Pittsburgh Sleep Quality Index (PSQI) [26], range 0–21). The questionnaires were carried out at enrollment (baseline punctuation) and at 34–36 weeks of gestation (final punctuation). Abnormal scores were considered the 75th percentile of the baseline scores of each questionnaire in the usual care group, except for the WHO-5 questionnaire, which presents a previously reported cut-off point that defines optimum mental well-being as a score greater than 52 [27].

2.5. Sample Collection

In a subgroup of randomly selected participants from each study group (excluding those receiving corticosteroid treatment), the 24 h urinary cortisone and cortisol metabolites were measured at the baseline and final assessment and analyzed by a validated method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) [28]. The activity of 11β-Hydroxysteroid Dehydrogenase Type 2 was estimated by the cortisone/cortisol ratio.

2.6. Statistical Analysis

Clinical data are presented as mean (standard deviation (SD) or standard error (SE)), median (interquartile range (IQR)) or number (percentage), as appropriate. The methods of statistical analyses used for the comparison of clinical and perinatal characteristics included Student's *t*-test, ANOVA or ANCOVA with baseline adjustments for continuous variables and X^2 test for categorical variables. Differences were considered significant when p-value < 0.05. Statistical analyses were performed using the Statistical Package for the Social Sciences statistical software package version 27 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Study Population and Pregnancy Outcomes

Within these patients, after excluding those that did not provide lifestyle questionnaires to measure their anxiety and stress, mindful state and sleep quality, a population of 680 individuals was considered (n = 331 for Mediterranean diet, n = 349 for usual care), as reported in Figure 1.

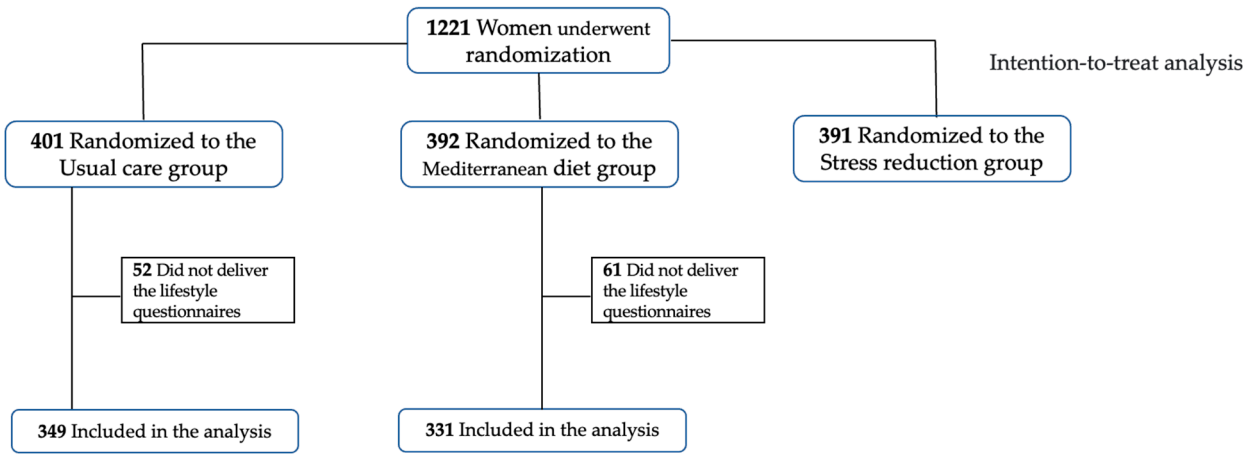

Figure 1. Flowchart of participants from the IMPACT BCN trial involved in the current study.

Baseline characteristics of the study population are shown in Table 1 with no differences between study groups. Pregnancy and perinatal outcomes are shown in Supplementary Table S1, with no significant differences between groups apart from the prevalence of SGA newborns, as reported in the main outcome of the trial [17].

Table 1. Baseline characteristics of women included in the study according to intervention groups (n = 680).

Characteristics	Usual Care n = 349	Mediterranean Diet n = 331	p Value
Age at recruitment (years)	37.1 (33.3–40.5)	37.3 (34.7–40.4)	0.28
Ethnicity			
White	281 (80.5%)	269 (81.3%)	0.80
Latin	50 (14.3%)	44 (13.3%)	0.70
Afro-American	6 (1.7%)	5 (1.5%)	0.83
Asian	6 (1.7%)	7 (2.1%)	0.70
Others	6 (1.7%)	6 (1.8%)	0.93
Socio-economic status [a]			
Low	20 (5.7%)	15 (4.5%)	0.48
Medium	106 (30.4%)	86 (26.0%)	0.20
High	223 (63.9%)	230 (69.5%)	0.12
BMI before pregnancy (Kg/m^2)	23.7 (4.8)	24.0 (4.7)	0.60
BMI > 30 kg/m^2 before pregnancy	39 (11.2%)	38 (11.5%)	0.90
Medical history before pregnancy			
Autoimmune disease	48 (13.8%)	39 (11.8%)	0.44
Thyroid disorders	20 (5.7%)	29 (8.8%)	0.13
Chronic hypertension	15 (4.3%)	8 (2.4%)	0.18
Diabetes Mellitus	12 (3.4%)	16 (4.8%)	0.36
Psychiatric disorders	11 (3.2%)	8 (2.4%)	0.56
Chronic kidney disease	5 (1.4%)	6 (1.8%)	0.70
Obstetric history			
Nulliparous	143 (41.0%)	145 (43.8%)	0.46
Previous placental disease	68 (19.5%)	66 (19.9%)	0.88
Previous preterm birth	9 (2.6%)	10 (3.0%)	0.73
Use of assisted reproductive technologies	92 (26.4%)	85 (25.7%)	0.84
Cigarette smoking during pregnancy	28 (8.0%)	22 (6.6%)	0.49
Alcohol intake during pregnancy	8 (2.3%)	4 (1.2%)	0.27
Drug consumption during pregnancy	1 (0.3%)	2 (0.6%)	0.77
Sports practice during pregnancy	78 (22.3%)	71 (21.5%)	0.94
Yoga or Pilates during pregnancy	73 (20.9%)	63 (19.0%)	0.54

BMI: Body mass index. Data are expressed as median (IQR) or mean (SD) or n (%). [a] socioeconomical status: low (never work or unemployed >2 years), medium (secondary studies and work), high (university studies and work).

3.2. Effects of Mediterranean Diet on Stress, Anxiety, Well-Being, Sleep Quality and Mindful State

3.2.1. Life-Style Questionnaires

Table 2 displays baseline and final life-style questionnaire scores on stress, anxiety, well-being, sleep quality, and mindful state between study groups, and Table 3 reports the percentage of high/poor scores at the final assessment. Perceived stress, anxiety and poor sleep quality increased throughout gestation in all study groups (Figure 2). At the end of the intervention, participants in the Mediterranean diet group showed significantly lower levels of perceived stress as compared to patients undergoing usual care, as shown in Figure 2A (mean difference −0.85 (−1.63 to −0.06), p = 0.035). Similarly, the Mediterranean diet group presented significantly lower final anxiety scores compared to the non-intervention group (mean 13.6 ± 0.4 SE vs. 15.8 ± 0.5, p < 0.004) (Figure 2B), with a lower frequency of high anxiety scores (n = 58, 17.9% vs. n = 87, 25.4%, p = 0.020), as reported in Table 3. Aligned with the previous findings, women's sleep quality improved following the Mediterranean

diet intervention compared to controls (PSQI mean 7.0 ± 0.2 SE vs. 7.9 ± 0.2 SE, $p = 0.001$) (see Table 2 and Figure 2C).

Table 2. Changes in maternal anxiety, well-being, sleep quality, and mindful state evaluated at baseline and final evaluation according to intervention groups.

		Within-Group Mean Changes		*p* §	Between-Group Changes
		Usual Care *n* = 349	MedDiet *n* = 331		MedDiet vs. Usual Care Difference (95% CI)
Perceived stress scale score	Baseline †	16.3 ± 7.8	15.9 ± 7.6		
	Final ‡	17.0 ± 0.4 *	15.9 ± 0.4	0.035	−0.85 (−1.63 to −0.06)
State-trait Anxiety Inventory (anxiety)	Baseline †	14.1 ± 8.8	12.9 ± 8.3		
	Final ‡	15.8 ± 0.5 **	13.6 ± 0.4 *	0.004	−1.35 (−2.28 to −0.43)
State-trait Anxiety Inventory (personality)	Baseline †	15.8. ± 9.0	14.2 ± 7.9		
	Final ‡	15.8 ± 0.5	14.0 ± 0.5	0.100	−0.68 (−1.48 to 0.13)
WHO Five Well-being index	Baseline †	62.7 ± 17.3	67.5 ± 15.2		
	Final ‡	62.9 ± 0.9	66.6 ± 0.8	0.587	0.51 (−1.32 to 2.33)
Pittsburgh Sleep Quality Index	Baseline †	6.7 ± 2.4	6.4 ± 2.1		
	Final ‡	7.9 ± 0.2 **	7.0 ± 0.2 **	0.001	−0.73 (−1.15 to −0.31)
FFMQ 1: Observation	Baseline †	23.3 ± 5.9	24.2 ± 5.6		
	Final ‡	24.0 ± 0.3	24.6 ± 0.3	0.729	0.12 (−0.57 to 0.81)
FFMQ 2: Description	Baseline †	32.1 ± 5.5	32.7 ± 4.8		
	Final ‡	31.7 ± 0.3	32.4 ± 0.3	0.273	0.35 (−0.27 to 1.37)
FFMQ 3: Awareness	Baseline †	31.3 ± 6.0	31.3 ± 6.3		
	Final ‡	30.6 ± 0.4 *	30.0 ± 0.4 **	0.280	−0.51 (−1.43 to 0.41)
FFMQ 4: Non-judgmental	Baseline †	29.9 ± 5.6	30.1 ± 5.2		
	Final ‡	30.0 ± 0.3	30.0 ± 0.3	0.994	0.00 (−0.64 to 0.64)
FFMQ 5: Non-reactivity	Baseline †	22.5 ± 4.8	22.6 ± 4.8		
	Final ‡	22.9 ± 0.2	22.5 ± 0.3	0.091	−0.55 (−1.05 to 0.08)

MedDiet: Mediterranean diet; FFMQ Five Facet. Mindfulness questionnaire. † Baseline values are observed means ± SD. ‡ Final values are baseline-adjusted (least-squares) means ± SE and comparison among groups obtained with ANCOVA analysis. * $p < 0.05$ and ** $p < 0.001$ final from baseline comparison. § ANCOVA analysis.

Table 3. Frequency of women high maternal stress, poor well-being and sleep quality questionnaires score at final evaluation according to intervention groups.

Final Scores	Usual Care	Mediterranean Diet	*p* Value
	n = 349	*n* = 331	
Perceived Stress Scale score > p75	85 (24.4%)	80 (24.2%)	0.96
State-trait Anxiety Inventory (anxiety) score > p75 [a]	82 (23.9%)	75 (23.1%)	0.82
State-trait Anxiety Inventory (personality) score > p75 [a]	87 (25.4%)	58 (17.9%)	0.02
WHO Five Well-Being Index score < 52 [b]	95 (27.5%)	65 (19.8%)	0.02
Pittsburgh Sleep Quality Index score > p75 [c]	62 (21.8%)	44 (16.8%)	0.14

Data are expressed as *n* (%). High maternal stress/anxiety defined as Perceived Stress Scale and State-strait Anxiety Inventory scores above 75th percentile. Poor well-being defined as Five Well-Being Index score below 52. Poor sleep quality defined as Pittsburgh Sleep Quality score above 75th percentile. [a] Data available for 667 pregnancies. [b] Data available for 674 pregnancies. [c] Data available for 546 pregnancies.

Regarding the well-being questionnaire, 19.8% ($n = 65$) of women from the Mediterranean diet group presented with poor well-being as compared to 27.5% ($n = 95$) in the control group ($p = 0.02$), revealing better well-being (see Table 3 and Figure 3). No significant differences between groups were observed with the mindful state questionnaire (Table 2). Changes in key foods and nutrient intake during intervention are shown in Supplementary Tables S2 and S3.

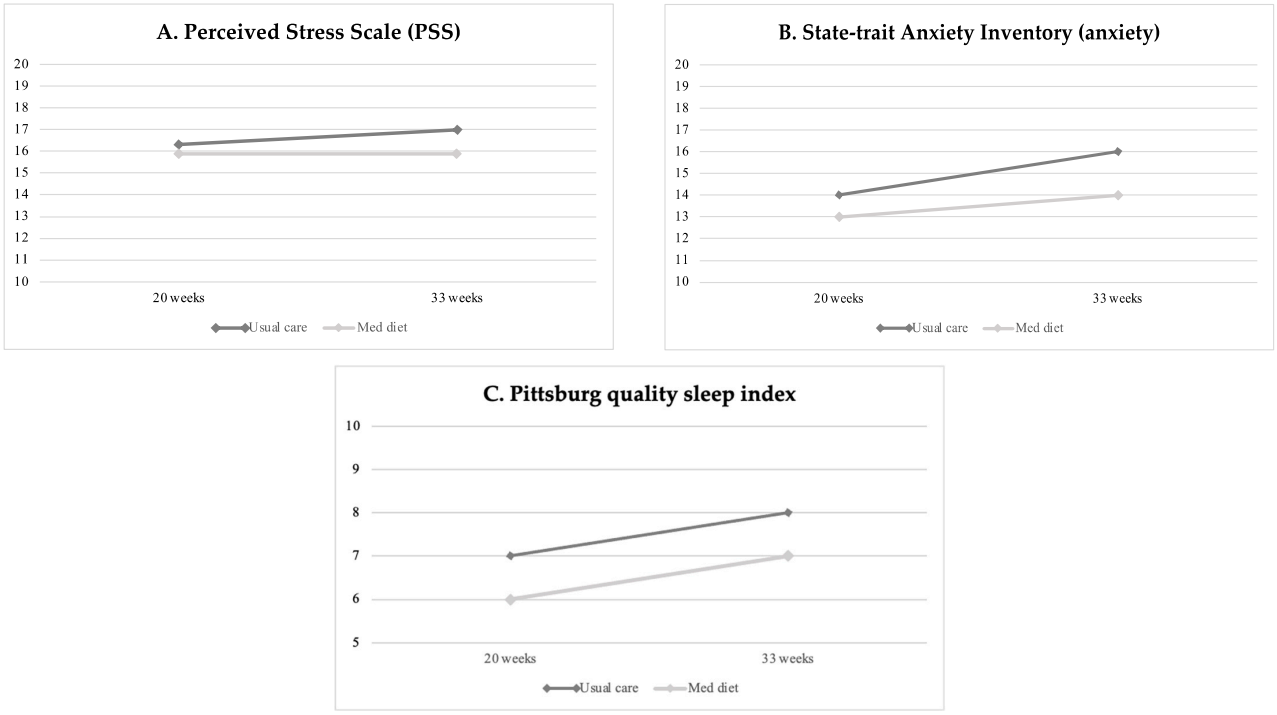

Figure 2. Changes in maternal stress (**A**), anxiety (**B**) and sleep quality (**C**) at baseline (20 weeks of gestation) and final (33 weeks) evaluation according to intervention groups.

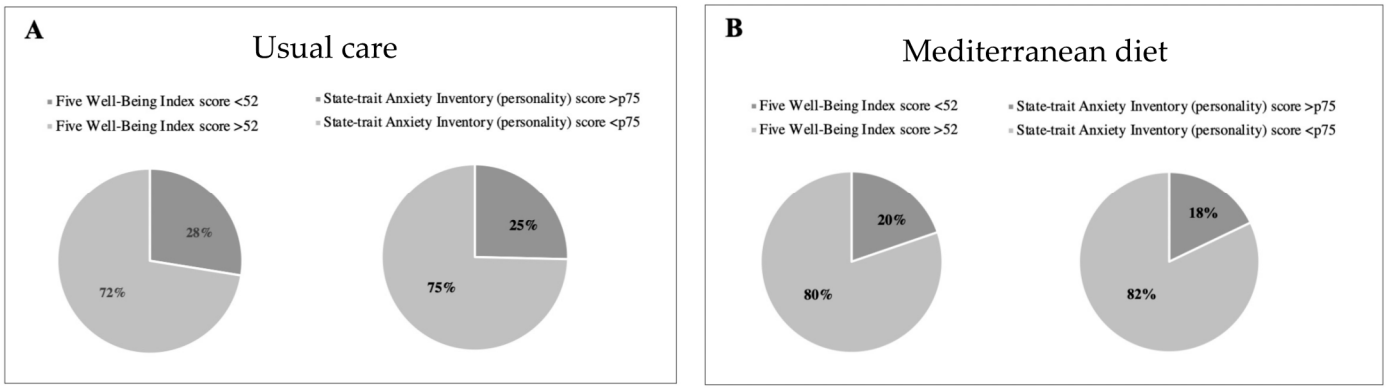

Figure 3. Percentage of high- vs. low-stress participants, and poor vs. good well-being (WHO-5) according to intervention groups. High stress is shown in dark grey color and defined as a State-trait Anxiety Inventory (STAI) personality score above 75th percentile in Usual care (**A**) and Mediterranean diet group (**B**). Poor well-being is shown in in dark grey color and defined as a Five Well-Being Index WHO score below 52.

3.2.2. Cortisol Assessment

The baseline 24 h urinary cortisone/cortisol ratio in 106 participants was similar between groups and increased during gestation. This increase was more pronounced in the Mediterranean diet group compared to usual care (mean 1.7± SE 0.1 vs. mean 1.3 ± SE 0.1, $p < 0.001$) (Table 4). At final assessment, Mediterranean diet participants showed higher levels of total cortisone concentration (mean 134.7± SE 8.3 vs. mean 111.5 ± SE 7.7, $p = 0.012$) and percentage (mean 2.9± SE 0.1 vs. mean 2.4 ± SE 0.1, $p = 0.002$), and lower levels of the 5β-tetrahydrocortisone/Cortisone (mean 16.8 ± SE 1.2 vs. mean 21.4 ± SE 1.4, $p = 0.032$) compared to the control group.

Table 4. Differences in urinary 24 h cortisol, cortisone and other related metabolites at baseline and final evaluation according to intervention group (n = 106).

		Within-Group Mean Changes		p §	Between-Group Changes
		Usual Care n = 52	MedDiet n = 54		MedDiet vs. Usual Care Difference (95% CI)
Total Cortisone/Total Cortisol	Baseline †	1.0 ± 0.6	1.2 ± 0.8		
	Final ‡	1.3 ± 0.1 **	1.7 ± 0.1 **	0.015	0.26 (0.05 to 0.47)
Total cortisol	Baseline †	89.9 ± 42.6	81.6 ± 36.1		
	Final ‡	89.8 ± 4.8	84.9 ± 5.3	0.619	2.66 (−7.83 to 13.16)
Total cortisol %	Baseline †	2.0 ± 0.8	2.0 ± 0.8		
	Final ‡	2.1 ± 0.1	2.0 ± 0.1	0.536	−0.08 (−0.33 to 0.17)
5β-tetrahydrocortisol	Baseline †	823.1 ± 419.3	734.4 ± 304.2		
	Final ‡	777.8 ± 54.6	766.3 ± 55.3	0.279	64.9 (−52.60 to 182.42)
5β-THF/Cortisol	Baseline †	10.0 ± 5.2	10.9 ± 5.0		
	Final ‡	9.1 ± 0.6	9.6 ± 0.7	0.774	0.19 (−1.13 to 1.52)
Total cortisone	Baseline †	85.6 ± 52.5	87.0 ± 50.1		
	Final ‡	111.5 ± 7.7 *	134.7 ± 8.3 **	0.012	24.3 (5.45 to 43.3)
Total cortisone %	Baseline †	1.9 ± 1.0	1.9 ± 0.7		
	Final ‡	2.4 ± 0.1 **	2.9 ± 0.1 **	0.002	0.47 (0.18 to 0.78)
5β-tetrahydrocortisone %	Baseline †	2185.2 ± 1189.3	1961.1 ± 973.2		
	Final ‡	2209.3 ± 171.2	2196.5 ± 184.4	0.627	111.0 (−336.96 to 558.99)
5β-THE/Cortisone	Baseline †	29.8 ± 15.5	26.3 ± 14.8		
	Final ‡	21.4 ± 1.4 **	16.8 ± 1.2 **	0.032	−3.39 (−6.49 to −0.30)

5β-THF/Cortisol: 5β-tetrahydrocortisol/Cortisol; 5β-THE/Cortisone: 5β-tetrahydrocortisone/Cortisone. † Baseline values are observed means ± SD. ‡ Final values are baseline-adjusted (least-squares) means ± SE and comparison among groups obtained with ANCOVA analysis. * $p < 0.05$ and ** $p < 0.001$ final from baseline comparison. § ANCOVA analysis.

4. Discussion

In this randomized clinical trial that involved pregnant women at high risk for an SGA newborn, an intervention based on MedDiet significantly reduced maternal anxiety and stress and improved well-being and sleep quality. These effects were revealed by self-reported stress questionnaires and biomarkers, as reflected by the increased estimated activity of a cortisol-deactivating enzyme.

Interest in mental health and care has grown exponentially in recent years and associations between healthy dietary patterns and mental health parameters have been reported. Jacka et al. conducted a randomized controlled trial to investigate the efficacy of a dietary intervention based on the MedDiet for the treatment of symptoms related to major depressive episodes in subjects with Major Depressive Disorder, independently of other factors such as physical activity, smoking habit, or weight loss [29]. The MedDiet group showed significantly greater improvements in symptoms of depression compared to the control group. In addition, other studies have evidenced that a lower incidence of depression incidence was significantly correlated with increasing adherence to MedDiet [7]. Additionally, in the PREDIMED study, a preventive effect for depression was found for the MedDiet in participants with type 2 diabetes [9]. Specifically, participants with type 2 diabetes allocated to the MedDiet supplemented with nuts group showed a 40% lower risk of depression compared to the control arm.

However, the evidence about the effects of dietary interventions on mental health during pregnancy is limited. Our study reveals that following the MedDiet during pregnancy is associated with a reduction in maternal anxiety/stress, together with an increase in the cortisol-deactivating enzyme. These findings are in line with previous data. In a recent study, Papandreou et al. conducted a randomized clinical trial with 40 pregnant women incorporating MedDiet recommendations into the Clinical Decision Support Systems, showing an improvement in nutritional status and reduction in health-related anxiety and depression [30]. Similarly, a longitudinal study with 152 pregnant women showed that

higher adherence to the MedDiet was inversely associated with anxiety and directly associated with well-being [16]. Moreover, these associations were significant for some key foods of the MedDiet, specifically whole-grain cereals, fruits and vegetables, extra-virgin olive oil and nuts [16], food sources of dietary antioxidants whose consumption was encouraged during the intervention in our study. Aligned with our findings, other healthy dietary patterns promoting healthy foods not based on the MedDiet were associated with lower depression during pregnancy [31–33]. Nevertheless, in observational and cohort studies with pregnant women, some specific foods have been identified as protective against mental disorders (including depression and anxiety), including whole-grain cereals, fruits, and beans. In contrast, other foods are associated with higher risk, including ultra-processed foods such as pastries, red and processed meat, margarine, and artificial juices [16,34]. Additionally, it has been postulated that levels of depression tend to increase throughout pregnancy, highlighting the importance of structured dietary interventions to improve overall diet quality during pregnancy [33,35].

In addition to its beneficial effects on anxiety and stress, our study first demonstrates an improvement in maternal well-being and sleep quality with MedDiet. The association between higher MedDiet adherence and subjective well-being has been found in observational studies [36]. In the case of sleep quality, a longitudinal study with 150 pregnant women assessed the association between MedDiet adherence and the Pittsburgh Sleep Quality Index, showing an association between higher MedDiet adherence and better sleep quality at 16- and 34-week's gestation, results aligned with our findings [37]. It should also be considered the burden that women go through during pregnancy may affect their mental health; research often does not recognize the multiple competing demands on women, specifically during pregnancy. However, to our knowledge, the present study is the first randomized clinical trial with a structured intervention based on a MedDiet adapted to pregnancy to evaluate well-being and sleep quality.

Several biological mechanisms have been postulated regarding the relationship between diet and mental health. First, it should be noted that the MedDiet is an easy-to-follow dietary pattern and is not only a healthy diet but also promotes a healthy lifestyle, including cultural and lifestyle elements such as conviviality, seasonality, traditional recipes, physical activity, and culinary activities [38]. These behavioral changes related to lifestyle may also have a therapeutic benefit [29]. Second, the role of diet in mental health may be mediated by inflammatory and oxidative stress pathways [12,13], the modulation of gut microbiota [39] and brain plasticity [40]. A low production of brain-derived neurotrophic factor, a peptide implicated in synaptic plasticity and neuronal survival, has been observed in patients with depression [41]. Moreover, reduced brain-derived neurotrophic factor levels were observed in pregnant women with low sleep quality, as measured by the Pittsburgh Sleep Quality Index, compared to pregnant women with good sleep quality [42]. Interestingly, in a sub-group of the PREDIMED study, significantly higher plasma levels of brain-derived neurotrophic factor were observed in participants allocated to the MedDiet supplemented with nuts group compared to the control arm, whose secretion may be also modulated by diet [43]. The fatty acid profile of the MedDiet, rich in polyunsaturated fatty acids, may also promote mental health, as low polyunsaturated fatty acid intake (mainly omega-3 fatty acids) has been associated with several mental outcomes, including depression [44,45]. Thus, several dietary components, including nutrients and bioactive compounds, are required for healthy brain function and mental health, including the synergic effect between components. Therefore, dietary interventions promoting a healthy dietary pattern rather than a single nutrient may have greater benefits for mental health [46].

Important implications regarding the mental health of the mother may be expected, including a potential benefit during the postpartum period. Maternal mental health alterations, principally anxiety, are associated with several adverse outcomes for both the mother and the offspring, including postnatal depression, pre-term birth and the poor cognitive and behavioral development of the infants [47–50]. Additionally, the estimated prevalence of anxiety disorders across the perinatal period is around 21% [51]. Our results

highlight the need for anxiety and stress screenings during pregnancy, nutritional education, and referrals for evaluation and treatment if necessary. Further research is needed to characterize the impact of the MedDiet on mental health during pregnancy, including the underlying mechanisms, specifically oxidative stress, and the potential benefits for the offspring's mental health. If confirmed, the MedDiet could become an early intervention strategy for the prevention of mental disorders [52].

The major strengths of the present study include a very well-characterized population of pregnant women who followed a structured intervention in a randomized clinical trial. Moreover, the use of different validated questionnaires with clinical applicability to assess mental stress, well-being and sleep quality provided rigor and validity to the results of the study, as well as the ability to analyze various stress-related biomarkers in a subgroup of patients with the aim of measuring stress in an empirical way. The use of validated questionnaires and biomarkers may mitigate the potential misclassification of self-reported data, along with the inherent risk of inaccuracies in the measurements.

The study has some limitations. Firstly, the trial was not designed for this purpose, although maternal stress, well-being and sleep quality were prespecified in the study protocol and assessed from the beginning of the study. Secondly, we were not able to assess long-term dietary intake, including measuring diet before pregnancy or the dietary changes from the beginning of the pregnancy. Most women were of white ethnicity and middle to high socio-economical level; hence, the results should not be extrapolated to other populations with different characteristics. These findings should be considered preliminary and require replication, including reseatch involving other study populations and an evaluation of the underlying mechanisms of action.

5. Conclusions

In conclusion, a MedDiet intervention significantly reduces maternal anxiety and stress, as well as improving well-being and sleep quality during gestation. Considering the increasing importance of the role of mental health during pregnancy, these findings might imply the promotion of a pregnancy-adapted MedDiet among pregnant women as a powerful public health strategy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu15102362/s1, Table S1. Pregnancy and perinatal outcome of women included in the study; Table S2. Changes in dietary key foods' intake and Mediterranean diet adherence evaluated at baseline and final visits according to intervention groups; Table S3. Changes in nutrient intake and Mediterranean diet adherence evaluated at baseline and final visits according to intervention groups.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets used and/or analyses during the current study are available from the corresponding author on reasonable request.

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Table SUPPL 1. Pregnancy and perinatal outcome of women included in the study (n=680).

Characteristics	Usual care n=349	Mediterranean diet n=331	p value
Gestational age at recruitment (weeks)	20.8 (0.7)	20.8 (0.6)	0.64
Pregnancy complications			
Preeclampsia	32 (9.2%)	19 (5.7%)	0.09
Mild	26 (7.5%)	13 (3.9%)	0.05
Severe	6 (1.7%)	6 (1.8%)	0.93
Prenatally diagnosed SGA	31 (8.9%)	18 (5.4%)	0.08
Threatened preterm labor	8 (2.3%)	11 (3.3%)	0.42
Preterm premature rupture of membranes	9 (2.6%)	8 (2.4%)	0.88
Stillbirth	1 (0.3%)	0 (0.0%)	0.33
Delivery outcome			
Gestational age at delivery (weeks)	39.3 (1.9)	39.4 (1.8)	0.21
Preterm birth	20 (5.7%)	22 (6.6%)	0.63
Induction of labor	181 (52.0%)	163 (49.2%)	0.47
Mode of delivery			
Vaginal delivery	204 (58.6%)	174 (52.6%)	0.11
Cesarean section	111 (31.9%)	122 (36.9%)	0.17
Operative vaginal delivery	33 (9.5%)	35 (10.6%)	0.64
Maternal anesthesia[a]	327 (94.2%)	309 (93.4%)	0.63
Antibiotics during labor[b]	157 (45.2%)	166 (50.6%)	0.17
Delivery complications[c,d]	20 (5.8%)	29 (8.9%)	0.12
Neonatal outcome			
Female gender	163 (46.8%)	165 (49.8%)	0.43
Birthweight (g)	3219 (2817-3501)	3250 (2992-3520)	0.12
Birthweight (percentile)	40.8 (30.4)	42.7 (29.1)	0.41
Small for gestational age	75 (21.6%)	46 (13.9%)	0.01
Severe SGA (<3rd centile)	31 (8.9%)	15 (4.5%)	0.02

Apgar 5 minutes <7[e]	1 (0.3%)	1 (0.3%)	0.10
pH umbilical artery[f]	7.2 (0.1)	7.2 (0.1)	0.25
Neonatal resuscitation	17 (4.9%)	12 (3.6%)	0.42
NICU admission	22 (6.3%)	18 (5.4%)	0.63

PE: preeclampsia; SGA: small for gestational age; NICU: Neonatal intensive care unit.
Data are expressed as median (IQR) or mean (SD) or n (%).
[a]Data available for 678 pregnancies.
[b]Data available for 675 pregnancies.
[c]Data available for 674 pregnancies.
[d]Placental abruptio, shoulder dystocia, postpartum hemorrhage, postpartum infection
[e]Data available for 664 pregnancies.
[f]Data available for 459 pregnancies.

Table SUPPL 2. Changes in dietary key-foods intake and Mediterranean diet adherence evaluated at baseline and final visits according to intervention groups.

		Mediterranean diet	**Usual Care**		**Mediterranean diet *vs.* Usual care**
				P[c]	*Difference (95% CI)*
Extra virgin olive oil – g/d	Baseline[a]	34.9 (18.7)	32.5 (19.4)		
	Final[b]	42.6 (0.96)**	39.3 (0.88)**	0.011	3.34 (0.78 to 5.90)
Refined olive oil – g/d	Baseline[a]	7.09 (13.7)	7.94 (15.2)		
	Final[b]	2.98 (0.70)**	5.56 (0.64)*	0.007	-2.57 (-4.43 to -0.72)
Total nuts – g/d	Baseline[a]	21.0 (21.2)	17.8 (18.0)		
	Final[b]	27.5 (1.16)**	23.2 (1.05)**	0.006	4.30 (1.23 to 7.38)
Vegetables – g/d	Baseline[a]	289.8 (129.8)	286.5 (127.8)		

	Final[b]	321.1 (6.40)**	298.4 (5.80)	0.008	22.8 (5.83 to 39.7)
Legumes – g/d	Baseline[a]	52.7 (41.8)	50.8 (34.8)		
	Final[b]	68.4 (2.76)**	63.4 (2.50)**	0.173	5.06 (-2.23 to 12.4)
Fruits – g/d	Baseline[a]	326.2 (169.9)	318.0 (162.2)		
	Final[b]	372.2 (11.2)**	340.8 (10.2)	0.039	31.3 (1.65 to 61.1)
Refined cereals – g/d	Baseline[a]	62.6 (47.3)	63.6 (42.1)		
	Final[b]	37.7 (2.26)**	51.2 (2.07)**	<0.001	-13.5 (-19.5 to -7.45)
Whole grain cereals – g/d	Baseline[a]	41.9 (44.0)	35.4 (36.8)		
	Final[b]	55.7 (2.34)**	46.4 (2.12)**	0.003	9.36 (3.15 to 15.6)
Fish or seafood – g/d	Baseline[a]	72.0 (42.2)	72.4 (43.3)		
	Final[b]	89.6 (2.51)**	78.8 (2.89)*	0.001	10.9 (4.23 to 17.5)
Fat fish – g/d	Baseline[a]	14.9 (16.4)	15.3 (16.1)		
	Final[b]	26.9 (1.20)**	19.7 (1.09)**	<0.001	7.19 (4.01 to 10.4)
Lean meat – g/d	Baseline[a]	71.3 (38.5)	68.9 (37.1)		
	Final[b]	77.8 (2.4)**	72.2 (1.87)	0.043	5.61 (0.18 to 11.0)
Red meat – g/d	Baseline[a]	46.5 (35.0)	50.2 ()36.7		
	Final[b]	42.0 (1.76)*	46.2 (1.61)	0.079	-4.19 (-8.87 to 0.48)
Processed meat – g/d	Baseline[a]	32.0 (27.6)	33.6 (26.1)		
	Final[b]	31.7 (1.20)	30.0 (1.09)*	0.299	1.69 (-1.50 to 4.87)
Pastries, cakes, or sweets – g/d	Baseline[a]	38.0 (32.9)	42.5 (37.5)		
	Final[b]	33.2 (1.86)*	35.7 (1.68)**	0.315	-2.52 (-7.44 to 2.39)
Dairy products – g/d	Baseline[a]	337.5 (214.7)	322.6 (198.5)		
	Final[b]	431.5 (13.0)**	397.6 (11.7)**	0.053	33.9 (-0.38 to 68.2)
Mediterranean diet score	Baseline[a]	7.97 (2.50)	7.46 (2.62)		
	Final[b]	12.1 (0.12)**	7.86 (0.12)*	<0.001	4.26 (3.92 to 4.60)

[a]Baseline values are observed means (SD). [b]Final values are baseline-adjusted (least-squares) means (SE) and comparison among groups done with ANCOVA analysis. *P<0.05 and **P<0.001 final from baseline comparison. [c]ANCOVA analysis.

Table SUPPL 3. Changes in nutrients intake and Mediterranean diet adherence evaluated at baseline and final visits according to intervention groups.

		Mediterranean diet	**Usual Care**		**Mediterranean diet *vs.* Usual care**
				P[c]	*Difference (95% CI)*
Energy – kcal/d	Baseline[a]	2468 (520.8)	2420 (509.1)		
	Final[b]	2526 (27.4)*	2502 (24.9)*	0.517	24.0 (-48.6 to 96.6)
Protein – kcal/d	Baseline[a]	103.9 (25.4)	102.7 (26.2)		
	Final[b]	113.6 (1.47)**	108.2 (1.33)**	0.007	5.36 (1.47 to 9.24)
Carbohydrate – g/d	Baseline[a]	222.9 (62.1)	216.0 (58.1)		
	Final[b]	214.5 (3.01)	217.0 (2.74)	0.545	-2.47 (-10.4 to 5.52)
Fiber – g/d	Baseline[a]	33.8 (11.1)	32.9 (10.8)		
	Final[b]	36.4 (0.59)**	34.8 (0.54)*	0.054	1.55 (-0.02 to 3.12)
Total fat – g/d	Baseline[a]	128.8 (30.6)	127.0 (30.0)		
	Final[b]	134.8 (1.65)**	133.4 (1.51)**	0.530	1.41 (-2.98 to 5.79)
SFA – g/d	Baseline[a]	34.7 (10.3)	34.5 (9.41)		
	Final[b]	35.1 (0.53)	35.8 (0.48)*	0.343	-0.68 (-2.08 to 0.73)
MUFA – g/d	Baseline[a]	61.9 (15.0)	61.2 (15.2)		
	Final[b]	64.3 (0.81)*	63.9 (0.73)*	0.685	0.44 (-1.69 to 2.58)
PUFA – g/d	Baseline[a]	22.7 (8.38)	22.0 (7.81)*		
	Final[b]	25.3 (0.48)**	23.8 (0.44)*	0.018	1.53 (0.26 to 2.80)
α-Linoleic acid – g/d	Baseline[a]	14.8 (6.33)	14.5 (5.74)		

	Final[b]	16.6 (0.36)**	15.7 (0.33)*	0.090	0.83 (-0.13 to 1.78)
α-Linolenic acid – g/d	Baseline[a]	1.43 (0.65)	1.38 (0.60)		
	Final[b]	1.96 (0.05)**	1.59 (0.04)**	<0.001	0.37 (0.25 to 0.50)
EPA – g/d	Baseline[a]	0.16 (0.11)	0.16 (0.11)		
	Final[b]	0.23 (0.01)**	0.18 (0.01)**	<0.001	0.04 (0.02 to 0.06)
DHA – g/d	Baseline[a]	0.32 (0.25)	0.33 (0.26)		
	Final[b]	0.50 (0.02)**	0.39 (0.02)**	<0.001	0.10 (0.06 to 0.15)
Trans-FA – g/d	Baseline[a]	1.66 (1.22)	1.66 (1.13)		
	Final[b]	1.31 (0.06)**	1.55 (0.05)	0.003	-0.24 (-0.40 to -0.08)
Cholesterol – mg/d	Baseline[a]	311.8 (98.8)	332.0 (101.4)		
	Final[b]	344.1 (5.25)*	332.8 (4.78)	0.111	11.3 (-2.60 to 25.2)

[a]Baseline values are observed means (SD). [b]Final values are baseline-adjusted (least-squares) means (SE) and comparison among groups done with ANCOVA analysis. *P<0.05 and **P<0.001 final from baseline comparison.
[c]ANCOVA analysis.

STUDY 4

Nasopharyngeal microbiota profiling of pregnant women with SARS-CoV-2 infection.

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Nasopharyngeal microbiota profiling of pregnant women with SARS-CoV-2 infection

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We aimed to analyze the nasopharyngeal microbiota profiles in pregnant women with and without SARS-CoV-2 infection, considered a vulnerable population during COVID-19 pandemic. Pregnant women were enrolled from a multicenter prospective population-based cohort during the first SARS-CoV-2 wave in Spain (March-June 2020 in Barcelona, Spain) in which the status of SARS-CoV-2 infection was determined by nasopharyngeal RT–PCR and antibodies in peripheral blood. Women were randomly selected for this cross-sectional study on microbiota. DNA was extracted from nasopharyngeal swab samples, and the V3-V4 region of the 16S rRNA of bacteria was amplified using region-specific primers. The differential abundance of taxa was tested, and alpha/beta diversity was evaluated. Among 76 women, 38 were classified as positive and 38 as negative for SARS-CoV-2 infection. All positive women were diagnosed by SARS-CoV-2 IgG and IgM/IgA antibodies, and 14 (37%) also had a positive RT–PCR. The overall composition of the nasopharyngeal microbiota differ in pregnant women with SARS-CoV-2 infection (positive SARS-CoV-2 antibodies), compared to those without the infection (negative SARS-CoV-2 antibodies) ($p = 0.001$), with a higher relative abundance of the Tenericutes and Bacteroidetes phyla and a higher abundance of the *Prevotellaceae* family. Infected women presented a different pattern of microbiota profiling due to beta diversity and higher richness (observed ASV < 0.001) and evenness (Shannon index < 0.001) at alpha diversity. These changes were also present in women after acute infection, as revealed by negative RT–PCR but positive SARS-CoV-2 antibodies, suggesting a potential association between SARS-CoV-2 infection and long-lasting shift in the nasopharyngeal microbiota. No significant differences were reported in mild vs. severe cases. This is the first study on nasopharyngeal microbiota during pregnancy. Pregnant women with SARS-CoV-2 infection had a different nasopharyngeal microbiota profile compared to negative cases.

The upper respiratory tract is the major portal of entry for infectious droplets or aerosol-transmitted microorganisms. The barrier function of its mucosa and the regulation of the immune response are modulated by the microbiota, the communities of microorganisms that colonize all of the surfaces of the human body, participating in host physiological and pathological processes[1]. Evidence suggests that dysbiosis of the upper respiratory tract (nose and nasopharynx) microbiota modulates the host´s susceptibility to pathological conditions, such as acute respiratory tract infections[2,3].

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is transmitted through microdroplets and aerosols produced by sneezing, coughing, or speaking[4]. The virus penetrates the host through the upper airways, which represent the first defense to avoid infection. The microbiota of the respiratory system may play a role from initiation to progression of coronavirus disease (COVID-19)[5]. However, evidence on the relationship between the upper respiratory tract microbiota and SARS-CoV-2 infection is still scarce and discordant. A study on 56 COVID-19 patients reported differences in the composition of specific operational taxonomy units (OTUs), mostly belonging to Bacteroidetes and Firmicutes phyla, and a loss of complexity abundance networks in the most severe cases[6]. Similarly, other studies demonstrated differences in the Chao1 and Shannon indexes[7], with an age dependency of the pharyngeal profile[8]. In contrast, other authors could not find any differences in either bacterial richness/diversity or composition[9–11], even if patients with overt COVID-19 had a lower abundance of Proteobacteria and Fusobacteria phyla[10] and lower taxonomic richness[12].

Pregnancy is a unique physiological state in which all body systems participate, including hormonal, immune and metabolic pathways[13]. Recent evidence illustrated gut microbiota changes over the course of a healthy pregnancy[14]; nevertheless, other human niches with potential physiological effects have been poorly studied. SARS-CoV-2 infection during pregnancy is mostly asymptomatic or mild[15–17], but similar to other respiratory viruses, there is a greater risk of severe respiratory complications compared with nonpregnant women, particularly in late gestation[18]. The characteristics of nasopharyngeal microbiota in women with SARS-CoV-2 infection during pregnancy have not been investigated.

In this study, we aimed to study the impact of SARS-CoV-2 infection on the nasopharyngeal microbiota of pregnant women at the third trimester of pregnancy. We further investigated the potential differences in nasopharyngeal microbiota in women with active *versus* past infection determined by SARS-CoV-2 PCR or the presence of specific viral antibodies; as well as the asymptomatic *versus* symptomatic SARS-CoV-2 infection symptoms and also, in relation to antibody titers.

Methods

Study design. Pregnant women were selected from a large multicenter prospective population-based cohort study conducted from March 15 to May 31, 2020, in Barcelona, Spain[17], during the first SARS-CoV-2 wave in Spain: all women consecutively admitted in three hospitals for delivery were recruited. Nasopharyngeal swab detection of SARS-CoV-2 RNA by real-time polymerase chain reaction (RT–PCR) and microbiota study and peripheral blood for antibody detection were obtained in all participants at recruitment. All the women consecutively admitted in the hospitals were tested for SARS-CoV-2 infection and those that accepted to participate in the study were assigned to the positive or negative group according to the result of both RT-PCR and serological test (see laboratory diagnostic procedures for SARS-CoV-2 infection section). For this specific study focused on nasopharyngeal microbiota, 76 women were randomly selected from the prospective cohort[17] to study the nasopharyngeal microbiota (half of them positive, half negative).

The study was approved by the ethics committee at each of the three involved institution (Ethical Committee of Hospital Clínic, study number HCB/2020/0434, Ethical Committee of Hospital Sant Joan de Déu study number PIC-56-20), and informed written consent was obtained from all women.

All methods were carried out in accordance with relevant guidelines and regulations involving human participants and human samples were conducted in accordance with the 1964 Helsinki Declaration and its later amendments.

Data collection. Pregnancy and perinatal data were obtained from electronic medical records. COVID-19 symptoms were recorded using a structured questionnaire for all pregnant women, which included questions about risk factors and about any symptom suggestive of COVID-19 noticed between mid-February 2020 and the time of testing for SARS-CoV-2.

Sample collection and laboratory diagnostic procedures for SARS-CoV-2 infection. For each participant, nasopharyngeal swab samples for SARS-CoV-2 RNA RT–PCR were collected by the hospital's trained staff. Samples were collected in storage tubes (Micronics) with Zymo DNA/RNA Shield Lysis Buffer. RNA was extracted using the Quick-DNA/RNA Viral MagBead kit (Zymo) and the TECAN Dreamprep robot. Five microliters of RNA solution were added to 15 µl of rRT-PCR master mix (Luna Universal Probe One-Step RT–qPCR Kit; New England Biolabs, USA) and used for amplification of SARS-CoV-2 N1 and N2 regions, as well as the human RNase P gene as a control, as described in the CDC-006-00019 CDC/DDID/NCIRD/Division of Viral Diseases protocol released 3/30/2020. A SARS-CoV-2-positive result was considered if the Ct values for N1, N2 and RNase P were below 38. Samples discordant for N1 and N2 were repeated, and samples with a $Ct \geq 40$ for RNase P were considered invalid.

Blood samples were drawn from peripheral veins for each participant. Serum was separated by centrifugation at 1500*g* for 10 min at 4 °C, and samples were immediately stored at − 80 °C until processing. SARS-CoV-2 IgG and IgM/IgA antibodies were tested in all maternal samples using COVID-19 VIRCLIA® Monotest (Vircell Microbiologist, Granada, Spain). Indeterminate results were retested (VITROS® Immunodiagnostic Products Anti-SARS-CoV2 Total Tests, Ortho Clinical Diagnostics, Rochester, NY, USA) and classified as positive or negative. Likewise, all samples that were positive for IgM + IgA but negative for IgG in women reporting no symptoms suggestive of COVID-19 during the 10 weeks prior to testing were retested by a quantitative suspension array assay based on xMAP Luminex technology (Luminex Corporation, Austin, TX, USA)[19] and classified as positive or negative. A positive serological result was considered in the presence of any of the following: (1) seropositivity for IgG, (2) seropositivity for IgM + IgA in women with symptomatic COVID-19, or (3) seropositivity for IgM and/or IgA confirmed by two tests (Vircell and Luminex).

SARS-CoV-2 infection was defined either by positive RT–PCR in nasopharyngeal swabs or a positive serological result. Active infection was defined by a positive RT–PCR, while women with a negative RT–PCR but positive serological testing were defined as past infection. Among SARS-CoV-2-infected women, we defined as symptomatic those with at least one of the following symptoms: fever, dry cough, loss of taste or smell, dyspnea, headache, myalgias, diarrhea, sore throat and rash on skin or discoloration of fingers/toes. Thus, positive cases were subclassified as asymptomatic if no symptoms were reported, mild if there was at least one symptom compatible with the infection, or severe if symptoms suggestive of pneumonia (persistent fever and cough) or dyspnea were reported, which required hospital admission for surveillance[20].

DNA isolation and sequencing. DNA was extracted from nasopharyngeal swab samples using the ZymoBIOMICSTM 96 MagBead DNA Kit (Zymo Research) following manufacturer's instructions. The extraction tubes were agitated using Tissue lyser II (Qiagen, Hilden, Germany) at 30 Hz/s for 10 min.

After DNA extraction, the V3-V4 region of the 16S rRNA of bacteria was amplified using region-specific primers (For: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′, Reverse 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) (ref).

Specific amplicons were obtained following the PCR protocol: 3 min at 95 °C (initial denaturation) followed by 35 cycles: 30 s at 95 °C 30 s at 55 °C, and 30 s at 72 °C, and a final elongation step of 5 min at 72 °C. PCR products were purified using AMPure XP beads (Beckman Coulter, Nyon, Switzerland) with a 0.9 × ratio according to manufacturer's instructions. The above described primers contain overhangs allowing the addition of full-length Nextera barcoded adapters for multiplex sequencing in a second PCR step, resulting in sequencing ready libraries with approximately 450 bp insert sizes. In brief, 5 µl of the first PCR purified product were used as template for a second PCR with Nextera XT v2 adaptor primers in a final volume of 30 µl using the same PCR mix and thermal profile as for the first PCR but with only 8 cycles. 25 µl of the second PCR product were purified with SequalPrep normalization kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. Mock community DNA was included as positive control for library preparation (Zymobiomics Microbial Community DNA, ZymoResearch, Irvine, CA, USA) as well as negative control to control the amplification and sequencing environmental and cross-contaminations.

Libraries were eluted in 20 µl final volume and pooled for sequencing. The final pool was quantified by qPCR using Kapa library quantification kit for Illumina Platforms (Kapa Biosystems, SigmaAldrich, Saint Louis, MO, USA) on an ABI 7900HT real-time cycler (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). Sequencing was performed using Illumina MiSeq (2 × 300 bp) and v3 chemistry with a loading concentration of 10 pM. In all cases, 15% of PhIX control libraries were used to increase the diversity of the sequenced sample. Negative controls included sample collection buffer, DNA extraction, and PCR amplification steps, PCR products after both PCR steps were visualized by electrophoresis gel (1.5% agarose) with SYBR Safe (ThermoFisher Scientific, Waltham, MA, USA). No visible bands were observed.

Computational and statistical analysis. Data are presented as the mean (standard deviation, SD), median (interquartile range, IQR) or number (percentage), as appropriate. Statistical analysis for comparison of clinical and perinatal characteristics included the use of *Student's t test* or Mann–Whitney U tests and Pearson χ^2 test for continuous and categorical variables, respectively, to compare SARS-CoV-2-positive *vs.* SARS-CoV-2-negative women. Differences were considered significant when $p < 0.05$.

Raw demultiplexed forward and reverse reads were processed using the following methods and pipelines as implemented in QIIME2 version 2020.2 with default parameters unless stated[21]. DADA2 was used for quality filtering, denoising, paired-end merging and amplicon sequence variant calling (amplicon sequence variant, ASV, i.e., phylotypes) using the qiime *dada2 denoise-paired* method[22]. Q20 was used as a quality threshold to define read sizes for trimming before merging. Reads were truncated at the position when the 75th percentile Phred score felt below Q20: 284 bp for forward reads and 224 bp for reverse reads. The average of the amplicon size obtained was 404.09 bp (min = 261 and max = 432 m STD = 14.8) after merging of the paired end reads. Phylotypes were filtered to discard contaminant eukaryote DNA-derived amplicons using BLAST against the eukaryote database with a 90% identity cutoff. After quality filtering steps, the average sample size was 28,303 reads (min: 8,965 reads, max: 64,404X reads), and 4823 phylotypes were detected. Negative controls were used to detect environmentally derived contaminants. Taxonomic affiliation results revealed that most contaminant amplicons were either absent in most samples or were at least two orders of magnitude less abundant than in the negative control. From those detected phylotypes, 43 phylotypes assigned to 36 taxa at Level 7 (ASV) were detected in the samples. Taxa (n = 22) that presented differences between experimental groups were removed from the analysis to avoid contaminant noise from diversity and composition analyses. A complete list of these phylotypes is reported in Table S1.

ASVs were aligned using the *qiime alignment mafft* method[23]. The alignment was used to create a tree and to calculate phylogenetic relations between ASVs using the *qiime phylogeny fasttree* method[24]. ASV tables were subsampled without replacement to even sample sizes for diversity analysis using *the QIIME diversity core-metrics-phylogenetic* pipeline. The smallest sample size was chosen for subsampling. Jaccard, Bray Curtis and unweighted and weighted UniFrac distances[25] were calculated to compare community structures. Alpha diversity metrics calculated were observed OTU number (i.e., richness), Pielou's evenness index and Shannon index. Taxonomic assignment of ASVs was performed using a Bayesian classifier trained with the Silva database v.132 (i.e., 99% OTU database) using the *QIIME feature-classifier classify-sklearn* method[26].

The differential abundance of taxa was tested using two methods, ANCOM[27] and the Mann–Whitney non-parametric test on the relative abundance of taxa (total sum scale-TSS). Alpha diversity comparisons were performed using the Kruskal–Wallis nonparametric test. After Kruskal–Wallis, Conover's test with FDR

	N = 76
Age, years	31.1 (27.3–35.8)
Race or ethnic group	
White	48 (63.2%)
Latin-American	15 (19.7%)
Black	0 (0%)
Asian	8 (10.5%)
Maghreb	5 (6.6%)
Socioeconomical status[#]	25 (32.9%)
Low	7 (9.2%)
Medium	35 (46.1%)
High	34 (44.7%)
Pre-pregnancy body mass index, kg/m^2	24.4 ± 4.7
Smoking during pregnancy	8 (10.5%)
Relevant comorbidities	
Chronic hypertension	2 (2.6%)
Diabetes mellitus	2 (2.6%)
Obesity[†]	10 (13.2%)
Asthma	10 (13.2%)
Hypothyroidism	10 (13.2%)
Pregnancy history	
Nulliparous	43 (56.6%)
Assisted reproductive technologies	6 (7.9%)
Multiple gestation	1 (1.3%)
Gestational age at recruitment, weeks	39.5 (2.1)

Table 1. Baseline characteristics of the study population. Data are n (%) or median (IQR) or mean ± SD. [#]Low socioeconomic status defined as study level (low: no studies, primary; medium: secondary; high: university). [†]Obesity defined as body mass index > 30 kg/m^2.

Benjamini–Hochberg correction was added for pairwise comparison. Beta diversity distance matrices and ASV tables were used to calculate principal coordinates (PCoA) and construct ordination plots. The significance of groups in community structure was tested using PERMANOVA[28], and the Permdisp test was used to identify location *vs.* dispersion effects[28]. BiodiversityR version 2.11-1[29], PMCMR version 4.3[30], RVAideMemoire version 0.9-7[31], vegan version 2.5-5 packages[32], R software package version 3.6.0 and IBM SPSS 26.0 (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp) were used to conduct all the statistical analyses. Spearman correlations between IgG and IgM/IgA levels and microbial taxa were performed on the Calypso online platform[33] and then plotted using the ggplots v 3.1.1 package[34]. Linear discriminant analysis (LDA) effect sized (LEfSe) analysis was performed for biomarker discovery using a size-effect cutoff of 3.0 on the logarithmic LDA score in the Calypso online platform. All plots were performed using the mentioned packages and ggplot2, qiime2R v. 0.99, forcats v. 0.5.1, ggpubr v. 0.4.0 and RColorBrewer v. 1.1-3 packages.

Results

Study population and pregnancy outcomes. The baseline characteristics of the study population are shown in Table 1. The average of maternal age was 31.3 years (SD 5.9; minimum 18.7, maximum 43.5 years). No differences were reported between positive and negative SARS-CoV-2 infected women (Table S2). The clinical characteristics of the study population according to SARS-CoV-2 infection are shown in Table 2. Among women with SARS-CoV-2 infection, 20 (52.6%) reported the presence of at least one symptom, and the most common symptoms were dry cough and fever (29% and 26%, respectively). Of a total of 38 infected women due to positive antibodies, only 14 (36.8%) also had a positive RT–PCR. Although 7 women (18%) required hospital admission for severe SARS-CoV-2 infection, only one presented pneumonia. None of them required intensive care unit (ICU) admission.

Pregnancy and perinatal outcomes in women depending on SARS-CoV-2 status are reported in Table S3. None of the newborns were infected by SARS-CoV-2. No differences between antibiotic consumption during pregnancy or delivery were reported.

Nasopharyngeal microbiota in the overall study population. Pregnant nasopharyngeal microbial communities were dominated by the Firmicutes phylum (34.7 ± 8.4%), followed by Proteobacteria (26.1 ± 11.7%) and Actinobacteria (20.2 ± 10.5%) (Figure S1). At the genus level, *Corynebacterium* (14 ± 11.4%) and *Staphylococcus* (9 ± 8.2%) were the most abundant genera (Figure S1).

	SARS-CoV-2 negative (n = 38)	SARS-CoV-2 positive (n = 38)	*p*
Symptoms compatible with SARS-CoV-2 infection within the last 10 weeks			
None	38 (100%)	18 (47.4%)	< 0.001
Fever ≥ 37.7 °C	0 (0%)	10 (26.3%)	< 0.001
Dry cough	0 (0%)	11 (28.9%)	< 0.001
Loss of taste or smell	0 (0%)	9 (23.7%)	0.001
Difficulty breathing or shortness of breath	0 (0%)	7 (18.4%)	0.005
Myalgia	0 (0%)	4 (10.5%)	0.040
Diarrhea	0 (0%)	3 (7.9%)	0.077
Fatigue	0 (0%)	4 (10.5%)	0.040
Sore throat	0 (0%)	1 (2.6%)	0.314
At least two symptoms or anosmia	0 (0%)	14 (36.8%)	< 0.001
At least three symptoms or anosmia	0 (0%)	13 (34.2%)	< 0.001
Presence of fever, cough and dyspnea	0 (0%)	6 (15.8%)	0.011
Diagnosis of SARS-CoV-2 infection			
RT-PCR positive	0 (0%)	14 (36.8%)	< 0.001
IgM/A and/or IgG for SARS-CoV-2 positive	0 (0%)	38 (100%)	< 0.001
IgM/A for SARS-CoV-2 positive	0 (0%)	26 (68.4%)	< 0.001
IgG for SARS-CoV-2 positive	0 (0%)	30 (78.9%)	< 0.001
Hospital admission for COVID-19	0 (0%)	7 (18.4%)	0.005
Pneumonia	0 (0%)	1 (2.6%)	0.314

Table 2. Clinical characteristics of the study population subdivided according to SARS-CoV-2 infection. Data are n (%). *SARS-CoV-2* severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), *RT-PCR* reverse transcriptase polymerase chain reaction.

SARS-CoV-2-infected pregnant women showed differences in nasopharyngeal microbiota. The structure of the nasopharyngeal microbial population was different between pregnant women with and without SARS-CoV-2 infection (Adonis based on unweighted UniFrac distance, F = 1.36, $p = 0.001$) (Fig. 1A). To further explore which taxa were specifically related to the infection, the ANCOM test identified 2 phyla and 18 genera whose relative abundance differed between positive and negative cases (Fig. 1B, C). At the phylum level, the microbiota of infected women was enriched in members of the Bacteroidetes and Tenericutes phyla (Fig. 1B, Table S4). At the genus level, microbial shifts were related to an increase in the relative abundance of several groups from the *Prevotellaceae* family, including the *Prevotellaceae NK3B31 group*, *Prevotella_1*, *Prevotella_9* and unclassified ASV from this family (Table S5). In addition, other genera from the *Ruminococcaceae* and *Lachnospiraceae* families, such as *Ruminococcaceae UCG-014*, *Ruminococcus 2*, *Ruminococcus torques* group, *Suboligranumlum* and *Faecalibacterium,* were also found to be enriched in SARS-CoV-2-infected pregnant women compared to uninfected women. Regarding the phylum Bacteroidetes, uninfected women displayed a lower relative abundance of the *Porphyromonadaceae* uncultured genus, *Parabacteroides* and *Rikenellaceae* RC9 group than those who were infected. These genera were also overrepresented in infected nasopharyngeal women, as reported by LEfSe analysis (Fig. 1C).

Alpha diversity analysis showed that SARS-CoV-2-infected pregnant women harbored a higher number of observed ASVs ($p < 0.001$) and a higher Shannon diversity index ($p < 0.001$) in their nasopharyngeal microbial populations than negative women (Fig. 1D). Indeed, Bacteroidetes and Tenericutes phyla, both enriched in nasopharyngeal microbiota of infected women, were also positive associated with higher alpha diversity indexes (Spearman correlation $p < 0.05$) (Figure S2), while lower abundance of Actinobacteria phylum was related to both, higher Shannon index (rho = − 0.58, $p < 0.001$, $q < 0.001$) and higher number of Observed ASV (rho = − 0.34, $p = 0.003$, $q = 0.004$) indexes, mainly due to the negative relation of *Corynebacterium* genus to both diversity indexes (not shown).

Differences in nasopharyngeal microbiota persist in women with a past SARS-CoV-2 infection. To gain more insight into the duration of the alterations in nasopharyngeal microbiota related to SARS-CoV-2 infection, we performed a comparison between SARS-CoV-2-infected mothers with an active infection (positive result in the RT–PCR at sample collection) and those with a past infection (negative result in the RT–PCR but positive antibodies) (Fig. 2). The effect of viral infection on both alpha and beta diversity was comparable in those women with a past infection, as both infected populations showed a similar nasopharyngeal microbiota profile. Beta-diversity analysis based on the unweighted UniFrac distance showed that women with a SARS-CoV-2 infection were clustered together independently of their infectious status at recruitment (Fig. 2A). Indeed, only the *Deinococcus-Thermus* ($q = 0.021$) phylum, with minor representation in nasopharyngeal microbiota, was enriched in women with a past infection. The phylum Actinobacteria was diminished in mothers with a past infection compared to control women ($q = 0.007$), suggesting the possible alteration of the nasopharyngeal microbiota after viral infection (Figure S3). No significant differences were observed at the genus

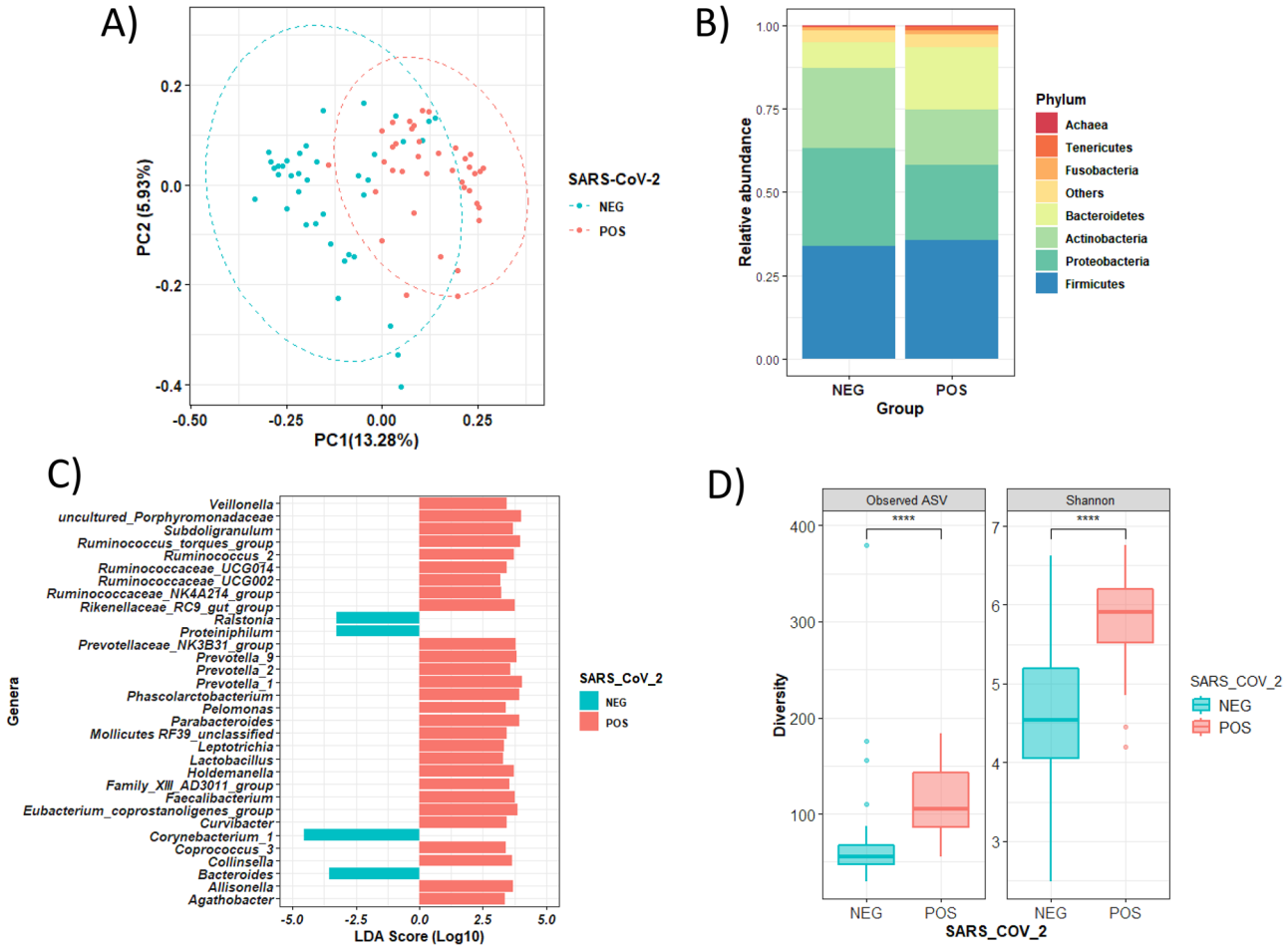

Figure 1. The nasopharyngeal microbiota of pregnant women is altered by SARS-CoV-2 infection. (**A**) Principal coordinates analysis (PCoA) ordination plot based on unweighted UniFrac distances according to SARS-CoV-2 infection. Each point corresponds to a sample. (**B**) Barplots showing the composition of the nasopharyngeal microbiota of the population in healthy (NEG) and SARS-CoV-2-infected (POS) pregnant women. Phyla with a relative abundance lower than 0.5% and Cyanobacteria were grouped as "Others" for plotting. (**C**) LDA effect size (LEfSe) analysis showing the genera that most discriminate both health conditions (infected *vs.* no infected). An LDA score > 3 was considered a significant threshold. (**D**) Boxplots showing the differences in the alpha diversity measured as observed ASV (amplicon sequence variant) and Shannon indexes according to SARS-CoV-2 infection. Statistical analysis of the differences between groups was calculated using the Kruskal–Wallis test with FDR correction for multiple comparisons. *POS* Positive result for SARS-CoV-2 (red), *NEG* negative result for SARS-CoV-2 (blue). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

level. Furthermore, while women who were negative for SARS-CoV-2 infection showed significantly lower diversity (observed ASV and Shannon index) than both women with a past infection ($q = < 0.001$ and $q < 0.001$, respectively) and those with an active infection ($q < 0.001$; $q = 0.001$), no differences were found between infected groups ($q = 0.964$; $q = 0.545$) (Fig. 2B).

SARS-CoV-2 antibody concentrations are associated with shifts in nasopharyngeal microbiota composition. Both IgG and IgM/A levels were associated with the pregnant nasopharyngeal microbiota (Fig. 3). In terms of alpha diversity, both IgG and IgM/A demonstrated a positive correlation ($p < 0.001$) with diversity indexes (Fig. 3A). Furthermore, a positive relation was observed between IgM/A and the proportion of Bacteroidetes (rho = 0.52, $p < 0.001$, $q < 0.001$) and Tenericutes (rho = 0.45, $p < 0.001$, $q = 0.001$), as was expected due to the differences in these phyla between positive and negative pregnant women (Fig. 3A). However, the results also revealed a negative relation between IgM/A concentration and the Actinobacteria phylum (rho = − 0.36, $p = 0.002$, q = 0.017) and *Corynebacterium* genus (rho = − 0.31, $p = 0.006$, $q = 0.075$). At the genus level, IgM/A was positively correlated with *Faecalibacterium* (rho = 0.36, $p = 0.001$, $q = 0.025$), *Subdoligranulum* (rho = 0.43, $p < 0.001$, $q = 0.003$), *Prevotellaceae*_UCG003 (rho = 0.32, $p = 0.005$, q = 0.064) and *Prevotella_1* (rho = 0.42, $p < 0.001$, $q = 0.004$) as well as several groups from the *Ruminococcaceae* family, including the *Ruminococcus_gauvreauii_group* (rho = 0.41, $p < 0.001$, $q = 0.005$) and *Ruminococcus_1* (rho = 0.35, $p = 0.002$, $q = 0.033$) (Fig. 3B). Regarding IgG concentration, a positive association was found with the mentioned genera, including

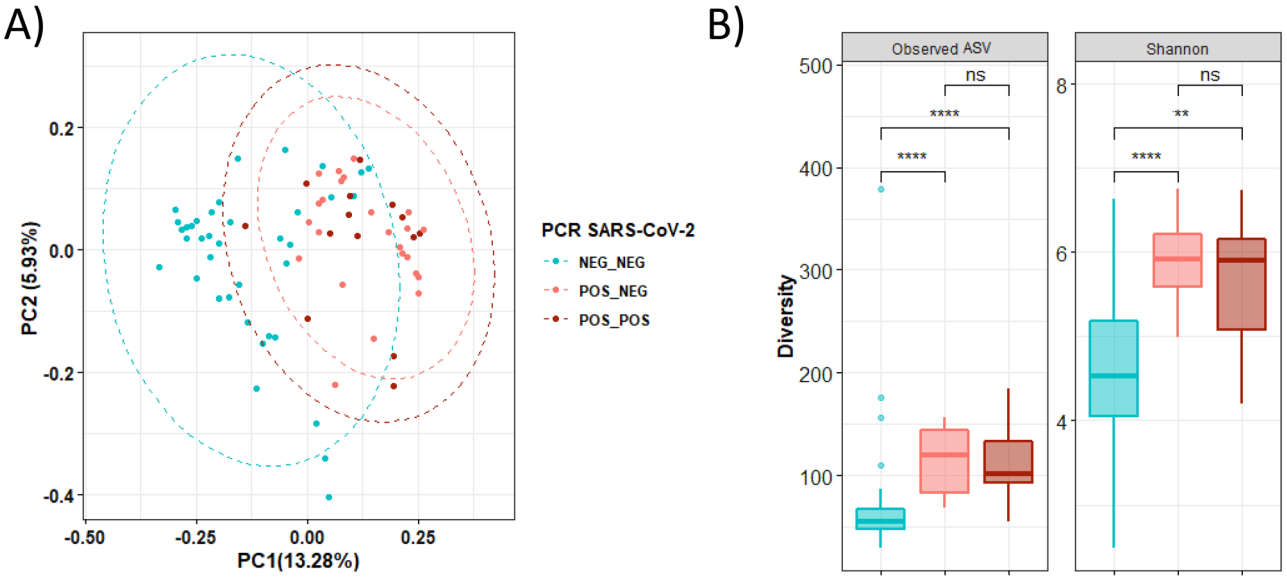

Figure 2. Similar nasopharyngeal microbiota in SARS-CoV-2-infected pregnant women with an active infection (positive RT–PCR and antibodies) *versus* a past infection (negative RT–PCR but positive antibodies). (**A**) Principal coordinates analysis (PCoA) ordination plot based on unweighted UniFrac distances according to the results of both serological and RT–PCR tests for SARS-CoV-2 infection (ADONIS F = 1.36, $p = 0.001$). Each point corresponds to a sample. (**B**) Boxplots showing the differences in the alpha diversity measured as observed ASV and Shannon indexes according to the results of both serological and RT–PCR tests for SARS-CoV-2 infection. POS_POS: Pregnant women with SARS-CoV-2 positive result for serological test and for nasopharyngeal RT–PCR (active infection); POS_NEG: SARS-CoV-2 positive result for serological test but negative nasopharyngeal RT–PCR (past infection); NEG-NEG: Noninfected pregnant women with SARS-CoV-2 negative serological and nasopharyngeal RT–PCR results. Statistical analysis of the differences between groups was calculated using the Kruskal–Wallis test with FDR correction for multiple comparisons. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

Prevotella_2 (rho = 0.39, $p = 0.001$, $q = 0.028$), *Prevotellaceae*_UCG003 (rho = 0.33, $p = 0.003$, $q = 0.110$), *Ruminococcus_torques*_group (rho = 0.38, $p = 0.001$, $q = 0.033$) and *Suboligranulum* (rho = 0.44, $p < 0.001$, $q = 0.007$), or uncultured *Porphyromonaceae* family members (rho = 0.5, $p < 0.001$, $q = 0.001$) (Fig. 3B).

The microbiota composition was similar in COVID-19 women with different clinical severities. Finally, we analyzed the relation with the severity of symptoms; no significant differences were found in terms of alpha diversity among the three severity groups (asymptomatic, mild and severe) (Figure S4); only with the inclusion of negative women was a slightly positive correlation observed between symptom severity numerical variables and both Shannon and observed ASV indexes. Similarly, while the ANCOM test revealed no differences between infected pregnant women with symptoms and those who remained asymptomatic, the Kruskal–Wallis test showed that nasopharyngeal microbiota from asymptomatic women harbored a higher relative abundance of *Enterococcus* (q = 0.004) and *Catenibacterium* (q = 0.014) and a lower proportion of *Ruminococcaceae_uncultured* women (q = 0.023).

Discussion

The present study reports differences in the nasopharyngeal microbial structure and composition of SARS-CoV-2-infected *versus* noninfected pregnant women. SARS-CoV-2-positive pregnant women showed differences in microbiota richness and evenness, with a higher relative abundance of Bacteroidetes (mainly due to the higher abundance of the *Prevotellaceae* family) and Tenericutes phyla. Additionally, we showed that these microbial changes were similar among women with past and present SARS-CoV-2 infection. No significant differences were reported in the most severe cases.

To our knowledge, this study is the first to describe the nasopharyngeal microbiota profile in SARS-CoV-2 infection during pregnancy. Previous studies in nonpregnant COVID-19 individuals reported a similar general microbial composition in the nasopharyngeal tract, with Firmicutes, Bacteroides, Proteobacteria and Actinobacteria as the most relevant phyla[6,9]. In the present study, a different nasopharyngeal profile was reported in pregnancies infected by SARS-CoV-2. Our findings are in agreement with several studies reporting differences in patients with this infection; however, the results are contradictory: Nardelli et al.[10] reported differences in beta diversity, with a reduction in Proteobacteria and Fusobacteria phyla in a subsample of 18 COVID-19 patients. A significant reduction in alpha diversity was reported in 19 COVID-19 patients who were hospitalized in the ICU, whereas no changes were found due to SARS-CoV-2 positivity[7]. In contrast, Ventero et al.[6] did not find any difference in the richness index between positive and negative cases; only in those patients who later developed more severe COVID-19 symptoms was there a loss of network complexity with a higher relative abundance of

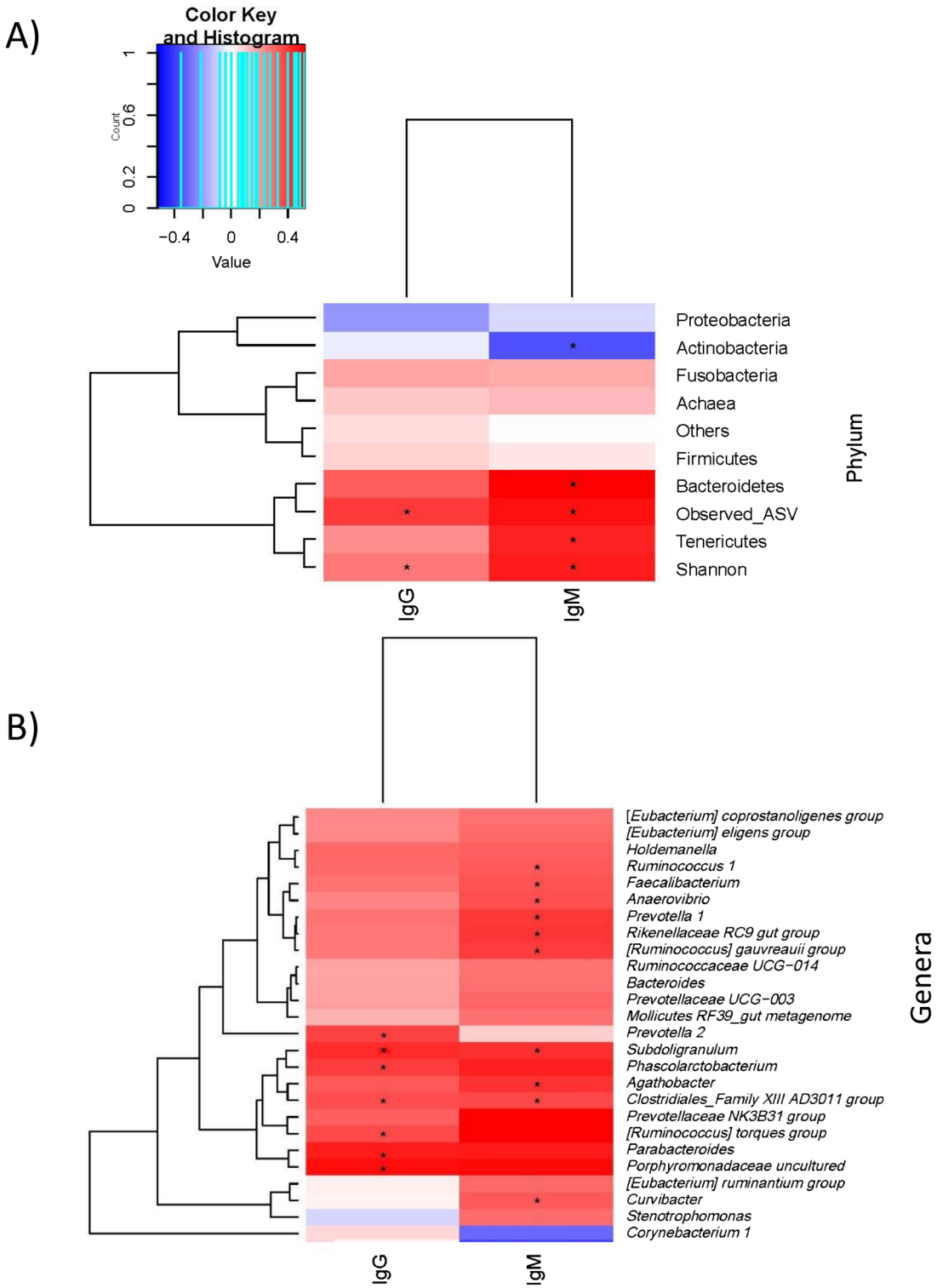

Figure 3. Several taxa of nasopharyngeal microbiota from pregnant women were related to the concentrations of both immunoglobulin M/A and G. Heatmap of the Spearman correlations between microbial taxa in the nasopharyngeal microbiome of pregnant women at the phylum (**A**) and genus levels (**B**) and the plasma concentration of immunoglobulin M/A (IgM/IGA) and G (IgG). At the phylum level, those phyla with a relative abundance lower than 0.5 and Cyanobacteria were grouped as "Others". Only those genera with significant associations with at least one of the analyzed immunoglobulins are shown. The significant associations are marked with an asterisk. The color of the cell represents the positive (red) or negative (blue) association.

the *Prevotella* genus. No differences in bacterial richness, diversity or composition between positive and negative SARS-CoV-2 patients were reported by De Maio et al.[9].

Such differences can be related to relatively small sample sizes, different populations and different severities of the condition, which requires several therapies.

Similar findings, such as a higher nasopharyngeal microbiota diversity and richness observed in SARS-CoV-2-infected pregnancies, have also been described in influenza-infected children compared to healthy children[35]. Other studies evaluating other viral infections, mostly influenza virus, have reported that the infection could change the diversity and composition of the nasopharyngeal bacterial community[3,36]. Moreover, in other parts of the respiratory tract, such as lung tissue, several authors reported an enrichment of pathogenic and commensal bacteria in COVID-19 patients[37–40]; in line with this, many authors agree that healthy lung tissue has a low density of microbial populations[41], and disorders in the microbiota would be characterized by enrichment of OTUs[40].

It can be hypothesized that respiratory microbial communities could be associated with SARS-CoV-2, without being possible to establish causality: infected patients had an altered respiratory tract microbiome with, in several cases, an increased abundance of OTUs. However, evidence is limited to studies with a relatively small sample size and different participant characteristics. Indeed, nor our study neither some with others with the same study design[6] was unable to stablish a causal link between SARS-CoV-2 and the alteration in these microbial communities.

This is the first study reporting that changes in the nasopharyngeal microbial community persisted after SARS-CoV-2 infection in pregnant women. This evidence supports the idea of lost-lasting effects of these changes after the acute phase of the infection. Since we did not have a baseline evaluation, we cannot ascertain whether changes in the microbiota were present before the infection; and thus, it is not possible to decipher the role of the SARS-CoV-2 as a potential cause of the observed alterations. However, we believe this is unlikely, considering that other respiratory infections have also been reported to induce changes in the nasopharyngeal microbiota[3,35,36]. An additional finding of this study was the association between taxa overrepresented in SARS-CoV-2-infected women and the levels of IgA/IgM, suggesting a potential relationship between the immune response and the microbiota[42]. Specifically, there was a negative association between IgA/IgM levels and the *Corynebacterium* genus, which is one of the main components of the nasopharyngeal microbiota[43] and has been related to a healthy condition in several studies[44] due to its potential capacity to compete with opportunistic pathogens[45]. These findings suggest that the microbiota alterations associated with SARS-CoV-2 could be mediated by the host immune system response.

Other studies have shown how maternal microbiota could change during pregnancy[14], with a potential role in the initial bacterial seeding of the neonate[46], which could also contribute to the immune system development during early life[47]. Not only at delivery but also during the gestational process, maternal microbiota has been proposed as a factor to drive fetal development and the susceptibility of the future infant to some diseases[48]. However, nothing is known about how the pregnancy and other related conditions could affect the nasopharyngeal microbiota. Therefore, the present study provided data about a potential association of COVID-19 with microbiota alteration but also provide data about the pregnant women nasopharyngeal microbiota.

In this study, we did not find any differences according to symptom severity. The small subgroup sample size and the high proportion of asymptomatic/mild infections may have hampered observing differences if these existed. Lee et al. reported in a nonpregnant population that several species from *Alloprevotella* and *Prevotella* were associated with influenza virus infection[3]. These taxa were also observed to be related to SARS-CoV-2 infection severity by Ventero et al.[6]. Moreover, overexpression of *Prevotella* proteins was related to an increase in the clinical severity of COVID-19[38]. In this study, we found a nonsignificant trend of higher relative abundance of the *Prevotella* genus and several groups from the Ruminococcaceae family.

Our study has some strengths and limitations that deserve comment. Among the strengths, to our knowledge, this is the first study of nasopharyngeal microbiota in pregnant women, providing data about this specific population and opening the door to future studies focused on them. Moreover, nasopharyngeal RT–PCR swab collection was always performed using a standardized procedure from trained medical staff at hospital admission, reducing potential bias before any treatment was started. Moreover, the population was very well characterized by SARS-CoV-2 infection status and COVID-19 symptoms. Nasopharyngeal samples are considered low-biomass samples, and specific positive and negative controls need to be introduced during sequencing to rule out potential contamination and bias due to the low microbial DNA samples. In this study, specific ASV were identified in the negative controls. After filtering process, we further identified specific anaerobic gut microbes, such *Ruminococcus* and *Faecalibacterium*, in the nasopharynx samples, although in lower proportions as other studies also reported to be present in the nasopharynx[49,50]. Furthermore, the butyrate production of those microbial genera would be associated with a reduction in olfactory function[50], which has been described as a COVID-19 symptom. Another potential explanation would be that the microbial database used as the curation of the open databases is critical for proper identification and reliable taxonomy assignment[51].

Among limitations, the relatively small sample size did not allow us to draw robust conclusions from subgroup comparisons; additionally, as there were no data on the upper respiratory tract microbiota during pregnancy, it was not possible to discern if changes were due only to the pregnancy status itself and if this could be considered a protective effect for viral infections to become more severe. Finally, future studies are warranted to compare these data with women of the same age but in a nonpregnant status.

Conclusions

In conclusion, the overall composition and diversity of the nasopharyngeal microbiota differed in pregnant women with and without SARS-CoV-2 infection. These changes were also present in women with a past infection. Further studies are needed to confirm our results and to evaluate the possible clinical implications of nasopharyngeal microbiota alterations in pregnancies complicated with SARS-CoV-2-CoV-2 infection.

Data availability

The datasets generated and/or analyzed during the current study are available at this link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA777915?reviewer=8agmagknr66hc31evjrnf7b8ui.

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Author contributions

F.Cro., F.C. and E.G. conceived and designed the study. M.D.G.R. and E.G. were responsible for the study protocol at each hospital and guaranteed the correct execution of the study. F.Cro. and F.C. were the supervisors at each hospital for day-to-day running of the study, including participant recruitment and data collection. R.P., M.L., I.C., and M.T. were responsible for recruitment, medical file revision and data collection at the hospitals involved. C.M.A. was the microbiologist responsible for nasopharyngeal SARS-CoV-2 RT–PCR data interpretation. N.E. and P.G. were responsible of laboratory procedures. B.C., P.G., M.S.R. and M.C.C. were responsible for microbiota bioinformatic analysis and interpretation. F.Cro. and M.S.R. did statistical analysis. F.Cro. and M.S.R. drafted the first version of the manuscript. E.G. was the principal investigator the project. All authors critically reviewed and approved the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

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STUDY 5

Effects of Mediterranean diet or Mindfulness Based-Stress Reduction during Pregnancy on Maternal Gut and Vaginal Microbiota. A sub analysis of the IMPACT BCN trial.

Selma-Royo M, Crispi F, Castro-Barquero S, Casas I, Larroya, M, Genero M, Paulés C, Benitez L, Youssef L, **Pascal R**, Dacal M, Nakaki A, Martín-Asuero A, Oller-Guzmán MT, Arranz A, Vieta E, Casas R, Estruch R, Gratacos E, Collado MC, Crovetto F.

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Effects of Mediterranean diet or Mindfulness Based-Stress Reduction during Pregnancy on Maternal Gut and Vaginal Microbiota. A sub analysis of the IMPACT BCN trial.

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Clinical Trial Information:

1) Data of Institutional Review Board (HCB-2016-0830) approval: December 16, 2016

2) Date of registration in govtrials: April 19, 2017

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6) URL of the registration site: https://clinicaltrials.gov/ct2/show/NCT03166332

7) Data sharing information

a. Will individual participant data be available (including data dictionaries)? Yes.

b. What data in particular will be shared? Deidentified participant data and data dictionary.

c. What other documents will be available (e.g., study protocol, statistical analysis plan, etc.)? Study protocol and analysis plan.

d. When will data be available (start and end dates)? Data will be available on the day of publication.

e. How will data be shared (including with whom, for what types of analyses, and by what mechanism)? The data will be shared to researchers whose proposed specified use of the data has been approved by the Ethical Committee of the authors' institute, with a signed data access agreement. To request data, one should contact francesca.crovetto@sjd.es via email.

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Tweetable Statement

First description of the effect of maternal lifestyle interventions during pregnancy on gut and vaginal microbiota.

Short version of the title

Maternal lifestyle and microbiota during pregnancy.

AJOG at a glance

Why was this study conducted?

We aimed to assess the effect of specific structured lifestyle interventions during pregnancy, based on Mediterranean diet or Mindfulness-Based Stress Reduction, on women's microbiota, both gut and vaginal niches.

What are the key findings?

Pregnant women, who followed a nutritional intervention based on Mediterranean diet or a stress-reduction intervention based on mindfulness, showed a different gut microbiota profile as compared to usual care, with minimal effects on vaginal microbiota.

What does this study add to what is already known?

Lifestyle interventions during pregnancy modulate maternal gut microbiota, with negligible effect on vaginal microbiota.

ABSTRACT

Objective: To investigate whether interventions during pregnancy based on a Mediterranean diet or Stress Reduction influence maternal microbiota.

Study design: A randomized clinical trial including 1221 pregnant women allocated into: a Mediterranean diet intervention, a Stress Reduction program, or no intervention. In a random subsample (n=267), maternal fecal/vaginal samples were collected at the end of the interventions (34-36 weeks), and gut/vaginal microbiota composition and diversity were profiled by 16S rRNA amplicon-gene sequencing (Illumina technology).

Results: Both Mediterranean diet and Stress Reduction interventions increased the microbial richness of maternal gut microbiota, and Stress Reduction also the microbial diversity. Women in both interventions harbored gut microbiota with higher abundance of healthy-associated genera such as *Blautia* or *Faecalibacterium* for Mediterranean diet, and *Lachnospiraceae* or *Ruminococcaceae* for Stress Reduction. A negligible effect was observed on vaginal microbiota.

Conclusion: Lifestyle interventions during pregnancy influence maternal gut microbiota, with a minimal effect on vaginal microbiota.

Keywords: Microbiota, Pregnancy, Mediterranean diet, Stress Reduction, Randomized clinical trial.

INTRODUCTION

The importance of gut microbiota for human health has become more evident since specific links between alterations of microbiota composition (*dysbiosis*) and several pathological conditions have been described[1]. During pregnancy, many physiological changes such as metabolism, hormonal cascades, immunological events etc., occur to promote the development of the fetus and prepare the mother for childbirth and lactation. Recent studies have reported changes in vaginal[2] and gut[3] microbiota during pregnancy. Maternal microbiota represents the most important microbial source for the neonatal microbiota colonization process and thus, could also be involved in the neonatal development with implications on the future adult health[4], including the risk of obesity, metabolic syndrome, diabetes, and allergy-related problems[5]. Even though gestational and perinatal factors that shape the maternal microbiota are yet to be truly defined, environmental conditions such as diet and lifestyle seem to be relevant in adult microbiota composition[6].

Although few studies reported influences of maternal lifestyle during pregnancy on microbiota composition, all of them came from observational studies with no application of structured interventions[7,8]. Pregnancy is a unique period in which health care interventions to improve the mother's well-being can affect both maternal and fetal health. Thus, strategies during pregnancy may provide an opportunity to positively affect the outcome of the pregnancy itself together with mother and offspring health[9].

In this context, we aimed to assess the effect of structured lifestyle interventions during pregnancy, based on Mediterranean diet or Mindfulness-Based Stress Reduction, on pregnant women's microbiota, both gut and vaginal niches.

MATERIALS AND METHODS

Study design, Population and Ethics

Pregnant women were randomly selected from the IMPACT BCN (***I**mproving **M**others for a better **P**ren**A**tal **C**are **T**rial **B**ar**C**elo**N**a*), which was a parallel, randomized clinical trial conducted at BCNatal (Hospital Clínic and Hospital Sant Joan de Déu), a large referral center for maternal-fetal and neonatal medicine in Barcelona, Spain. Details of the trial are provided in the protocol[10], approved by the Institutional Review Board (HCB-2016-0830) before any participant enrolment, and registered (ClinicalTrials.gov identifier NCT03166332) before any outcome occurred. All individuals who agreed to participate provided written informed consent before randomization.

Participants were screened for eligibility during routine second trimester ultrasound scans (19-23.6 weeks of gestation) under the criteria of the Royal College of Obstetrics and Gynaecologists for being at high risk of developing a small-for-gestational-age (SGA) newborn[11]. Participants who accepted to join the trial, were randomly assigned 1:1:1 to one of the three study groups: a Mediterranean diet supplemented with extra-virgin olive oil and walnuts; a stress reduction intervention based on Mindfulness; or usual care without any intervention (control group - no intervention). The primary outcome of the trial was the prevalence of SGA newborns and results can be found elsewhere[9]. The impact of these interventions on gut/vaginal microbiota composition and diversity were prespecified exploratory outcomes in the study design[10].

Sample size

Among participants recruited for the IMPACT BCN trial, 20% of each study group were randomly selected to evaluate the maternal gut and vaginal microbiota at the end of the

intervention (34-36 weeks' gestation), assuming a type I error of 5% and aiming for a power of 80% for finding any differences on the gut microbiota among involved participants. Thus, among the 1221 randomized pregnancies, 270 women were randomly selected among study groups (being n=90 women from each study group) for the study of maternal gut and vaginal microbiota.

Interventions and measurements

The interventions were nonpharmacological, based on counseling and behavioral training.

Mediterranean diet program

The dietary intervention aimed to change the general dietary pattern, instead of focusing on changes in single foods or macronutrients. All participants received extra-virgin olive oil (2L every month) and 15g of walnuts per day (450gr every month) at no cost. Dietitians conducted face-to-face interviews at enrollment, and monthly until the end of intervention (34-36 weeks' gestation). Additional details of the intervention are provided elsewhere[10].

Mindfulness-Based Stress Reduction program

The stress reduction program was based on the program described by Kabat-Zinn and later adopted by health institutions. It was consistent with previously described Mindfulness-Based Stress Reduction programs for adults: 8-week program of weekly 2.5 hour sessions, one full-day session, and daily home practice, with specific adaptation to pregnancy. The sessions included didactic presentations, formal 45-minute meditation practices with various mindfulness meditations, mindful yoga, body awareness and group discussion. Additional details of the intervention are provided elsewhere[10].

Usual care (non-intervention group)

Women randomized into this group received usual pregnancy care as per institutional protocols.

Data collection

Data of participants included in the study were pseudo-anonymized and entered an electronic case report form. All individuals included in the trial had a baseline visit (19 to 23 weeks) and a final visit (34 to 36 weeks) with a dietitian to assess a validated 151-item food-frequency questionnaire[12], and the 7-day dietary journal and the 17-item dietary of Mediterranean adherence assessment (score range: 0-17). All participants also provided self-report lifestyle questionnaires to measure their anxiety and mindful state *(State-trait Anxiety Inventory-STAI Anxiety and Personality*; *Perceived Stress Scale-PSS; WHO Five Well Being Index-WHO5*; *Five Facet Mindfulness Questionnaire-FFMQ.* During the last evaluation during gestation (34-36 weeks), maternal fecal and vaginal samples were also collected and stored at -80ºC until further analysis (**Figure 1A**). Perinatal results were recorded in the hospital database and medical discharge within 28 days after delivery.

Maternal fecal and vaginal sample collection and DNA extraction

Women fecal and vaginal samples were collected at 34-36 weeks of gestation using a sterile cotton-tipped swab and stored at -80ºC until analysis. All fecal and vaginal swab DNA was isolated using the MasterPure Complete DNA & RNA Purification Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions with modifications that included a bead-beater step and enzyme incubation to increase DNA extraction as described elsewhere[8].

Microbiota profiling: 16S rRNA amplicon sequencing

For the taxonomical profiling of the pregnant women's microbiota, the region V3-V4 from the 16S rRNA gene was sequenced using Illumina technology (Nextera XT Index Kit). Amplicons were checked with a Bioanalyzer DNA 1000 chip and libraries were sequenced using a 2x300pb paired-end run (MiSeq Reagent kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). During DNA extraction and PCR amplification, controls were included and sequenced.

Computational analysis of the resulted amplicons was performed using dada2 v.1.22.0 R package in both fecal and vaginal datasets independently. Quality-trimmed and filtering was assessed after quality examination and reads were trimmed at the 270th and 220th nucleotide in forward and reverse position, respectively. Additionally, primers were also removed during this initial trimming step with the other default parameters. Final reads were merged and chimeric sequenced removed to obtain the final amplicon sequenced variant (ASV) table. Taxonomic assignment was conducted using the Silva v138.1 database with the addition of the specie level classification for the vaginal dataset. Samples with less than 1000 reads were also removed from the final analysis in both fecal microbiota (n=3) and vaginal (n=1) datasets.

Statistical analysis

Clinical data are presented as mean (standard deviation, SD or standard error, SE), median (interquartile range, IQR) or number (percentage), as appropriate. Statistical analysis for comparison of clinical and perinatal characteristics included the use of ANOVA or ANCOVA

with baseline adjustment for continuous variables and χ^2 test for categorical variables. Differences were considered significant when *p-value <0.05.*

Extended description of the computational methods used for microbiota composition and diversity analysis are specified in **Supplementary data** (Materials and Methods).

RESULTS

Characteristics of the study population

Out of the 270 women randomly selected for this study, after quality filters based on the number of reads, 267 women were included in the microbiota analysis. Baseline and perinatal characteristics of the study populations are shown in **Tables I-II,** with no differences observed among the study groups. While participants in the three study groups reported similar baseline patterns of food and nutrient intake, at final assessment, significant differences were observed (see **Supplementary data: Tables SI, SII**), and final score of the 17-item Mediterranean Diet score significantly increased for the Mediterranean diet group (**Table SI**). Similarly, while no differences have been reported at recruitment, at the end of the intervention's participants in the Stress Reduction group had significantly higher scores on the mindful state-related questionnaire (**Supplementary data: Table SIII**).

Effect of the interventions on maternal gut microbiota

Overall, gut microbiota from pregnant women in the study was dominated by Firmicutes (mean: 65.36%; being *Peptoniphilus* the most relatively abundant genus, mean: 9.47%), followed by Bacteroidetes (mean: 22.71%, *Prevotella* as the main representative genus, mean: 5.34%) and Actinobacteria (mean: 7.04%, being *Corynebacterium* its more represented

genus, mean: 3.10%) (**Figure 1B**). Multivariate analysis based on Bray-Curtis distance (Adonis) revealed that overall structure of the gut microbiota was similar among groups (F=1.22, *p=0.078*) (**Figure 1C**), with pre-gestational body-mass-index (BMI) being a significant factor modulating the microbiota profile (F=1.99, *p=0.003*).

Principal Component Analysis revealed significant differences in the microbiota variation in the three study groups (*p=0.025* and *p=0.023* for Mediterranean Diet and Stress Reduction, respectively) (**Supplementary data: Figure S1)**. Furthermore, interventions modulated the microbiota composition, as showed in the canonical coordinate analysis (*p=0.018*) (**Figure 1D**). When the three groups are compared, mothers from the Stress reduction group showed an enrichment in Firmicutes (p=0.018, q=0.118) and Proteobacteria (p=0.006, q=0.076) phyla, compared to the rest of the groups (**Figure 1B)**. Due to the differential nature of both interventions, the effect of both dietary and stress reduction programs on microbiota modulation were assessed independently as described below.

Maternal gut microbiota and Mediterranean diet intervention

Women that followed the Mediterranean diet strategy showed significant higher microbial richness, observed ASV (*p=0.027*) and Chao1 index (*p=0.035*), as well as diversity (*p=0.042*), than those included in the usual care program (**Figure 1E**). Indeed, some genera were overrepresented in these women compared to those without intervention, despite this effect does not impact the overall beta-diversity of the gut microbial community (F=1.257, *p=0.118*). At genus level, LefSe analysis was performed to decipher the microbial markers representative of each group (**Figure 2A** and **2B**). Specific enrichments in *Faecalibacterium*, *Bifidobacterium, Bacteroides* and *Blautia* genera were found in mothers from the Mediterranean diet (**Figure 2A; Table III**), as well as a higher representation of short-chain

fatty acid (SCFA) producers' genera such as Ruminococcaceae and *Lachnospiraceae* families. On the contrary, microbiota from women who followed a usual care program was overrepresented by *Porphyromonas*, *Anaerococcus* and *Campylobacter* genera. Association of the microbial taxa with specific nutrients is reported in **Supplementary data** (text and Figure S2).

Maternal gut microbiota and Stress reduction intervention

Stress reduction program had an impact on the overall structure of the gut–microbiota (F=1.401, *p= 0.040* Adonis test on the Bray-Curtis distance). Indeed, this intervention had increased microbial richness measured through the number of Observed ASV (*p=0.035*) and Chao1 index (*p=0.056*), compared to the usual care strategy (**Figure 1E**), with no significant impact on microbial diversity assessed by Shannon index (*p=0.140*).

Adjusted models revealed higher relative abundance of Firmicutes (*p=0.045*, *q=0.194*) and Proteobacteria phyla (*p=0.062*, *q=0.200*) in this group compared to the Usual Care (**Figure 1B**, **Table IV**. At genus level, a higher representation of health-associated taxa including *Faecalibacterium* or *Christensenellaceae*_R7 group and SCFA producers' genera such as *Subdolonigranum*, and *Lachnospiraceae* and *Ruminococcaceae* were also observed (**Figure 2B**). Association of the microbial taxa with specific stress related questionnaires is reported in **Supplementary data** (text and Table S IV-V).

Effect of interventions on the vaginal microbiota of pregnant women

Vaginal microbiota was mainly characterized by the presence of *Lactobacillus* genus (median=93.7%, IQR=25.3) followed by in much less relative abundance by *Prevotella* (median=49.4%, IQR=32.6) and *Anaerococcus* (median=31.7%, IQR=15.8) genera (**Figure 3A**).

At species level, the samples showed a differential pattern of distribution based on the presence of different species (**Figure 3B**). The main group was clearly overrepresented by *Lactobacillus inner* and other by *Gardnerella vaginalis*. This distribution was also observed in the organization of the samples in the beta-diversity analysis based on Bray-Curtis distance (**Figure 3C**), which matched with the previously described community state types, that was observed as the main factor shaping the vaginal microbiota overall community (F=8.60, *p=0.001*). In the multivariate analysis based on Bray-Curtis distance, no significant effect of the intervention was found in the vaginal microbiota (F=0.89, *p=0.485*). Similarly, no effect of any intervention was observed in terms of alpha diversity (**Figure 3D**).

COMMENT

This sub analysis of the IMPACT BCN trial demonstrates a significant effect of lifestyle interventions during pregnancy, such as Mediterranean diet or Stress reduction, on the composition and/or diversity of gut microbiota of pregnant women. Both interventions were related to an increased microbial richness in the gut microbiota, with the Stress reduction strategy also impacting microbial diversity.

Maternal gut microbiota and Mediterranean diet intervention

Diet is one of the factors that most influences the microbiota[13] and is considered as an easy-to-be-implemented intervention targeting gut microbiota. A growing body of evidence has showed that a Mediterranean diet could modulate the gut microbiota, increasing its diversity and changing the proportion of some bacteria, compared to a Western food model[14]. In our study, participants in the Mediterranean diet group showed a microbiota enriched in

members of *Bacteroides*, *Blautia* and *Bifidobacterium* genera. *Blautia* has been consistently found to be decreased in some of some disorders such as obesity or inflammatory bowel diseases[15]. *Bifidobacterium* spp. are usually boosted by an increment in dietary fiber. Increased *Bifidobacterium* relative abundance in late pregnancy is reported to be related to progesterone levels. Since this genus is one of the main colonizers of the infant gut, it has been hypothesized that this could be one of the mechanisms on how the gestation could affect maternal microbiota with a role in the new-born colonization[16].

Women microbiota at delivery has been reported to be clustered according to diet[14]: *Blautia*, *Bifidobacterium* and several groups from *Ruminococcaceae* and *Lachnospiraceae* families were associated with a high intake of plant foods and some types of fatty acids typically of the Mediterranean diet. In fact, we also found a positive association between these families and the consumption of some of the key nutrients in this dietary model, including legumes and blue fish. Both aliments are rich in nutrients that have been reported to influence gut microbiota composition, including fibers[17] and n-3 Poly Unsaturated Fatty Acids from the legumes and the fish, respectively. Dietary counselling intervention also impacted the alpha-diversity of the pregnant women's microbiota, increasing both the diversity and richness of the gut bacterial community compared to the non-intervention group. These results are in line with previous studies on the general population following a Mediterranean diet and in some studies on the pregnant population[18].

Maternal gut microbiota and Stress reduction intervention

Stress and well-being are also considered determinants of gut microbiota. During pregnancy, stressors has been also described to impact the gut microbial composition and women exposed to prenatal stress had significantly different fecal microbial community composition

than non-stressed women[19]. Interestingly, we observed a significant effect of the Stress-reduction intervention on maternal gut microbiota composition, as this group showed significant higher alpha-diversity compared to usual care group. Gut microbiota from Stress intervention women harbored a higher relative abundance of *Faecalibacterium* and *Subdoligranulum* genera along with Lachnospiraceae and Ruminococcaceae families, in agreement with those studies[20] that found a reduction of these taxa associated with some mental disorders in general population. While most studies have been performed on the general population, few studies linked stressors and intestinal microbial composition during pregnancy[21]. Hechler *et al.,*[7] found in a pregnant women population that general perinatal stress indicators were negatively correlated with *Eubacterium* and *Oscillospira* genera, both associated with the capacity for butyrate production. This is the first time that a structured stress-reduction intervention shows an impact on microbial diversity and opens the door to the use of gut biomarkers as outcomes in psychological intervention trials, in the framework of precision psychiatry and psychotherapy[22].

Maternal vaginal microbiota

Usually, vaginal microbiome is considered a low-diversity environment dominated by *Lactobacillus* spp. and vaginal microbiota during gestation was described as less diverse with a higher predominance of *Lactobacillus* genus[23]. Previously, few studies explored the effect of dietary interventions targeting the vaginal microbiota. Houttu *et al.* report a reduction in the relative abundance of some pathobionts, such as *Ureaplasma*, in a group of overweight pregnant women after a fish oil and/or prebiotic consumption[24]. Others described that a general healthy diet pattern and the fiber intake or some micronutrients consumption can reduce the risk of developing bacterial vaginosis[25]. However, most of these analyses were

focused on the context of bacterial vaginosis. In our analysis, we found the previously described community state type in the maternal cohort; however, no effect of the interventions on vaginal microbiota was observed.

Strengths and limitations

As a strength, this study represents the first description from a randomized trial demonstrating an effect of lifestyle interventions during pregnancy on gut microbiota. As a limitation, we acknowledge that microbiota was not the primary outcome of the trial. Also, the relatively small sample size prevented a more comprehensive analysis on the association of microbiota and specific perinatal outcomes in each study group. In addition, the lack of samples collected before starting the intervention limited the analysis of longitudinal microbiota changes in each participant. Analyses were adjusted by maternal body mass index; however, we acknowledge that other potential confounders might have influenced the results. Finally, future studies are warranted to determine the influence of observed microbiota changes on perinatal and postnatal outcomes.

Conclusions

Lifestyle interventions during pregnancy influence maternal gut microbiota, with a minimal effect on vaginal microbiota. This is the first study that provides evidence on the effect of Mediterranean diet and stress reduction on maternal gut microbiota and highlights the maternal gut microbiota as potential therapeutic target. Future studies are warranted to assess the potential long-term benefits of microbiota as a therapeutic target.

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Author contributions

FCro, FC, MCC and EG conceived and designed the study. FCro, ML, CP, LB, LY, RP, MD, and AN had responsibility for day-to-day running of the trial including participant recruitment, data and sample collection. FCro and FC and guarantying the correct execution of the trial. SCB and TF were dietitians involved in the Mediterranean diet program, and RC and RE were responsible of the program. EV was responsible of the Mindfulness-Based Stress Reduction program. AMA and MTO were instructors of the Mindfulness-Based Stress Reduction program. AA was responsible of the nursing follow-up. MSR and MCC were responsible of the sample preparation, DNA extraction and microbiota analysis. MG gave her contribution to the database revision. FCro, MSR and IC did statistical analysis. FCro, MSR, FC, and MCC drafted the first version of the manuscript. All authors critically reviewed and approved the final version of the manuscript.

FIGURES' LEGEND

Figure 1. Effect of the interventions on the composition and diversity of gut microbiota from pregnant women. A) Diagram of the study design and the samples included in the study. **B)** Bar plots showing microbiota profile of the fecal microbiota at phylum level according to group. **C)** Principal coordinate analysis of the mothers according to microbiota beta diversity based on the Bray-Curtis distance. Significance of the intervention effect was assessed by Adonis test. **D)** Canonical coordinate analysis of the pregnant women according to gestational intervention. **E)** Effect of the gestational interventions on the alpha diversity of the gut microbiota measured as Observed ASV, Chao1 and Shannon index. Comparisons between the

interventional and the control groups were assessed by Mann-Whitney test after distribution testing.

Figure 2. Effect of individual interventions on gut microbiota from pregnant women. Linear discriminant analysis Effect Size analysis at genus level showing the genera that discriminate the microbial profile of the women according to groups in Mediterranean diet intervention (**A**) and Stress-reduction intervention (**B**). Only those genera with a LDA score >3 and a q-values <0.2 was included in the plot.

Figure 3. Association of vaginal microbiota of pregnant women with gestational intervention. A) Bar plots showing microbiota profile of the vaginal microbiota at genus level according to group. The top ten taxa are shown being the rest grouped as "Others". **B**) Heatmap showing the distribution of the relative abundance from the top 20 species on the vaginal microbiota of the pregnant women. Color legend show the community state type (CST) assessed by the VALENCIA tool (see methods). **C)** Principal coordinate analysis of the mothers according to microbiota beta diversity based on the Bray-Curtis distance. Significance of the intervention effect and CST was assessed by Adonis test. **D)** Effect of the gestational interventions on the alpha diversity of the vaginal microbiota measured as Observed ASV, Chao1 and Shannon index. Comparisons between the interventional and the control groups were assessed by Mann-Whitney test after distribution testing.

TABLES

Table I. Maternal characteristics of women included analysis depending on the group of intervention (n=267).

Characteristics	Usual care n=94	Mediterranean diet n=85	Stress reduction n=88	p value
Age at recruitment (years)	36.6 (33.0-40.4)	37.1 (34.8-40.3)	37.7 (35.1-40.4)	0.362
Ethnicity				
White	78 (83%)	67 (78.8%)	75 (85.2%)	0.534
Latin	12 (12.8%)	9 (10.6%)	11 (12.5%)	0.890
Afro-American	3 (3.2%)	4 (4.7%)	1 (1.1%)	0.384
Asian	1 (1.1%)	3 (3.5%)	1 (1.1%)	0.394
Others	0 (0%)	2 (2.4%)	0 (0%)	0.116
Socio-economic status[a]				
Low	5 (5.3%)	4 (4.7%)	3 (3.4%)	0.819
Medium	35 (37.2%)	37 (43.5%)	26 (29.5%)	0.161
High	54 (57.4%)	44 (51.8%)	59 (67%)	0.118
BMI before pregnancy (Kg/m^2)	23.4 (4.9)	24.7 (5.5)	23.4 (4.2)	0.143
Medical history				
Autoimmune disease	20 (21.3%)	8 (9.4%)	12 (13.6%)	0.077
Thyroid disorders	12 (12.8%)	7 (8.2%)	10 (11.4%)	0.612
Obesity (BMI ≥30)	9 (9.6%)	12 (14.1%)	6 (6.8%)	0.275
Psychiatric disorders	4 (4.3%)	4 (4.7%)	8 (9.1%)	0.324
Chronic hypertension	4 (4.3%)	4 (4.7%)	1 (1.1%)	0.361
Chronic kidney disease	2 (2.1%)	2 (2.4%)	1 (1.1%)	0.819
Obstetric history				
Nulliparous	51 (54.3%)	41 (48.2%)	355 (62.5%)	0.166
Previous SGA	18 (19.1%)	14 (16.5%)	8 (9.1%)	0.148
Previous preeclampsia	7 (7.4%)	6 (7.1%)	5 (5.7%)	0.885
Previous preterm birth	6 (6.4%)	8 (9.4%)	4 (4.5%)	0.436
Previous stillbirth	2 (2.1%)	3 (3.5%)	0 (0%)	0.225
Use of assisted reproductive technologies	26 (27.7%)	15 (17.6%)	28 (31.8%)	0.092
Cigarette smoking during pregnancy	5 (5.3%)	9 (10.6%)	5 (5.7%)	0.319
Alcohol intake during pregnancy	4 (4.3%)	3 (3.5%)	1 (1.1%)	0.193
Drugs consumption during pregnancy	1 (1.1%)	0 (0%)	0 (0%)	0.645
Sports practice during pregnancy	24 (25.5%)	14 (16.5%)	23 (26.1%)	0.156
Yoga or Pilates during pregnancy	16 (17%)	11 (12.9%)	16 (18.2%)	0.616

BMI: Body-mass index; SGA: Small for gestational age.

Data are expressed as median (IQR) or mean (SD) or n (%).

[a]Socioeconomical status: low (never work or unemployed >2years), medium (secondary studies & work), high (university studies & work)

Table II. Pregnancy and perinatal outcome of women included in the study according to intervention groups (n=267).

Characteristics	Usual care n=94	Mediterranean diet n=85	Stress reduction n=88	p value
Gestational age at recruitment (weeks)	20.8 (0.7)	20.8 (0.6)	20.9 (0.8)	0.581
Treatment during pregnancy				
Aspirin	27 (28.7%)	30 (35.3%)	29 (33%)	0.633
Heparin	5 (5.3%)	6 (7.1%)	2 (2.3%)	0.333
Antihypertensive drugs	2 (2.1%)	1 (1.2%)	2 (2.3%)	0.846
Progesterone	11 (11.7%)	6 (7.1%)	10 (11.4%)	0.526
Steroids	1 (1.1%)	2 (2.4%)	5 (5.7%)	0.173
Magnesium sulphate	1 (1.1%)	1 (1.2%)	2 (2.3%)	0.764
Iron	34 (36.2%)	29 (34.1%)	38 (43.2%)	0.432
Levothyroxine	9 (9.6%)	6 (7.1%)	11 (12.5%)	0.482
Antibiotics	9 (9.6%)	4 (4.7%)	10 (11.4%)	0.272
Antifungal	5 (5.3%)	4 (4.7%)	4 (4.5%)	0.968
Pregnancy complications				
Preeclampsia[a]	9 (9.6%)	6 (7.1%)	4 (4.5%)	0.430
Prenatally diagnosed SGA[a]	8 (8.5%)	4 (4.7%)	8 (9.1%)	0.484
Prenatally diagnosed severe SGA[a]	4 (4.3%)	2 (2.4%)	3 (3.4%)	0.780
Delivery outcome				
Gestational age at delivery (wks)	39.5 (1.4)	39.7 (1.2)	39.5 (1.6)	0.410
Preterm birth[a]	2 (2.1%)	4 (4.7%)	6 (6.8%)	0.302
Induction of labor[a]	49 (52.1%)	43 (50.6%)	44 (50%)	0.971
Mode of delivery[a]				
Vaginal delivery	53 (56.4%)	42 (49.4%)	57 (64.8%)	0.101
Operative vaginal delivery	10 (10.6%)	13 (15.3%)	7 (8.0%)	0.314
Cesarean section	31 (33%)	30 (35.3%)	23 (26.1%)	0.429
Maternal anesthesia[a]	87 (92.6%)	76 (89.4%)	77 (87.5%)	0.627
Antibiotics during labor[a]	43 (46.8%)	39 (45.9%)	42 (47.7%)	0.943
Delivery complications[a,b]	2 (2.1%)	2 (2.4%)	3 (3.4%)	0.996
Neonatal outcome				
Female[a]	43 (45.7%)	43 (50.6%)	39 (44.3%)	0.718
Birthweight (g)	3300 (2856-3632)	3230 (3000-3622)	3204 (2924-3550)	0.286
Birthweight (percentile)	47 (30.5)	44 (31.7)	41 (26.6)	0.448
SGA	16 (17%)	11 (12.9%)	11 (12.5%)	0.950
Apgar 5 minutes <7	0 (0%)	0 (0%)	0 (0%)	
pH umbilical artery	7.21 (0.1)	7.20 (0.1)	7.20 (0.1)	0.925
Neonatal metabolic acidosis[c]	6 (6.4%)	7 (8.2%)	6 (6.8%)	0.850
Neonatal resuscitation[a]	2 (2.1%)	2 (2.4%)	4 (4.5%)	0.569
Neonatal complications[a]	7 (7.4%)	9 (10.6%)	7 (8.0%)	0.708
NICU admission[a]	6 (6.4%)	3 (3.5%)	4 (4.5%)	0.669

PE: preeclampsia; SGA: small for gestational age; NICU: Neonatal intensive care unit.
Data are expressed as median (IQR) or mean (SD) or n (%).
[a]Data available for 266 pregnancies.
[b]Placental abruptio, shoulder dystocia, postpartum hemorrhage, postpartum infection
[c]Data available for 263 pregnancies.

12Table III. Multivariable model for the gut microbiota composition at phylum and genus level from pregnant women that followed the Mediterranean diet intervention as compared to usual care.

Phylum	**Coef (Usual Care)**	**SE**	**Prev.**	**p-value**	**q-value**
Firmicutes	-0.04	0.04	100	0.326	0.763
Epsilonbacteraeota	0.64	0.35	94.97	0.073	0.400
Fusobacteria	0.23	0.55	54.75	0.675	0.992
Lentisphaerae	**-0.52**	**0.17**	**17.32**	**0.003**	**0.069**
Actinobacteria	0.19	0.13	100	0.157	0.690
Euryarchaeota	-0.42	0.48	49.16	0.381	0.763
Bacteroidetes	-0.03	0.12	100	0.791	0.992
Synergistetes	-0.05	0.46	38.55	0.908	0.992
Cyanobacteria	-0.36	0.35	24.02	0.299	0.763
Verrucomicrobia	0.06	0.57	51.4	0.913	0.992
Proteobacteria	-0.25	0.28	98.32	0.377	0.763
Genus					
Lachnospiraceae_UCG-008	**-0.74**	**0.21**	**16.76**	**<0.001**	**0.048**
Victivallis	**-0.53**	**0.18**	**17.32**	**0.003**	**0.181**
Lachnospiraceae_NK4A136_group	**-1.46**	**0.5**	**71.51**	**0.004**	**0.187**
Lachnospiraceae_UCG-010	**-1.31**	**0.46**	**56.98**	**0.005**	**0.187**
Lactococcus	**-0.86**	**0.3**	**23.46**	**0.005**	**0.187**
Erysipelotrichaceae_UCG-003	-1.53	0.57	66.48	0.009	0.234
Collinsella	-1.23	0.49	79.89	0.012	0.246
Lachnospiraceae_UCG-004	-1.01	0.41	58.1	0.015	0.257
Ruminococcaceae_UCG-014	-1.57	0.67	63.13	0.020	0.302

Multivariable analysis was performed using Maaslin algorithm with the total sum scaling (TSS) normalization and log transformation. Pre-pregnancy body mass index was also included in the model as potential confounding factor. Only those genera with an FDR (q-value) <0.3 are shown in the table. Those taxa with significant association with the Mediterranean diet intervention are marked in bold.

Table IV. Multivariable model for the gut microbiota composition at phylum and genus level from pregnant women that followed the Stress reduction intervention as compared to usual care.

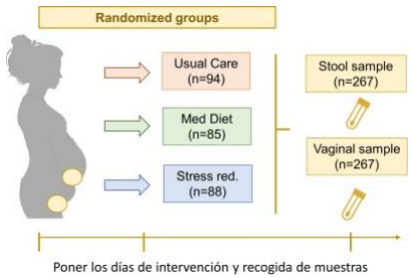
	Coef (Usual Care)	SE	Prev.	p-value	q-value
Phylum					
Firmicutes	**-0.09**	**0.04**	**100**	***0.045***	***0.194***
Epsilonbacteraeota	0.11	0.35	96.15	*0.761*	*0.811*
Fusobacteria	-0.26	0.49	58.79	*0.600*	*0.742*
Lentisphaerae	**-0.4**	**0.19**	**14.84**	***0.033***	***0.174***
Actinobacteria	**0.31**	**0.14**	**100**	***0.029***	***0.174***
Euryarchaeota	-0.4	0.43	46.7	*0.357*	*0.516*
Proteobacteria	**-0.56**	**0.3**	**98.35**	***0.062***	***0.200***
Bacteroidetes	**0.32**	**0.14**	**100**	***0.020***	***0.174***
Synergistetes	-0.25	0.44	41.76	*0.568*	*0.739*
Cyanobacteria	-0.56	0.34	28.57	*0.101*	*0.274*
Patescibacteria	-0.13	0.28	12.09	*0.647*	*0.764*
Tenericutes	**-0.85**	**0.29**	**13.74**	***0.004***	***0.116***
Verrucomicrobia	-0.7	0.56	58.79	*0.210*	*0.389*
Genus					
Ralstonia	**-2,43**	**0,36**	**43,41**	***<0.001***	***<0.001***
Lachnospiraceae_UCG-008	**-0,78**	**0,2**	**17,03**	***<0.001***	***0.017***
Lachnospiraceae_UCG-010	**-1,45**	**0,44**	**59,34**	***0,001***	***0,070***
Lachnospiraceae_NK4A136_group	**-1.54**	**0.49**	**73.63**	***0.002***	***0.086***
Lachnospiraceae_UCG-004	**-1.19**	**0.38**	**62.09**	***0.002***	***0.086***
Family_XIII_UCG-001	**-1**	**0.34**	**45.6**	***0.003***	***0.107***
Ruminiclostridium_6	**-1.3**	**0.48**	**46.7**	***0.008***	***0.190***
Anaerococcus	0.55	0.21	99.45	*0.011*	*0.226*
Haemophilus	-0.61	0.24	17.03	*0.013*	*0.227*
Butyricicoccus	-1.13	0.45	74.18	*0.013*	*0.227*
Collinsella	-1.28	0.5	80.22	*0.012*	*0.227*
Ruminococcaceae_UCG-014	-1.46	0.6	62.64	*0.016*	*0.247*
Lachnospira	-1.23	0.52	74.73	*0.020*	*0.265*
Ruminococcaceae_UCG-005	-1.09	0.46	73.63	*0.019*	*0.265*
Subdoligranulum	-1.17	0.49	85.71	*0.019*	*0.265*
Erysipelotrichaceae_UCG-003	-1.29	0.57	65.38	*0.024*	*0.267*
Slackia	-0.86	0.38	56.04	*0.023*	*0.267*
Porphyromonas	0.81	0.37	97.8	*0.029*	*0.308*

Multivariable analysis was performed using Maaslin algorithm with the total sum scaling (TSS) normalization and log transformation. Pre-pregnancy body mass index was also included in the model as potential confounding factor. Only those genera with an FDR (q-value) <0.3 are shown in the table. Those taxa with significant association with the Stress reduction intervention are marked in bold.

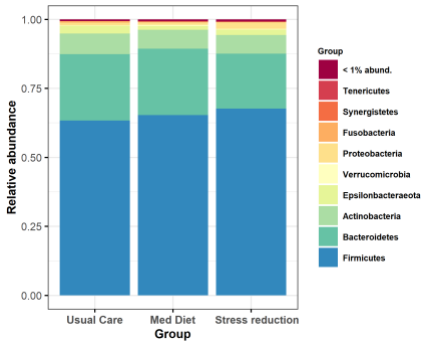
FIGURES

Figure 1.

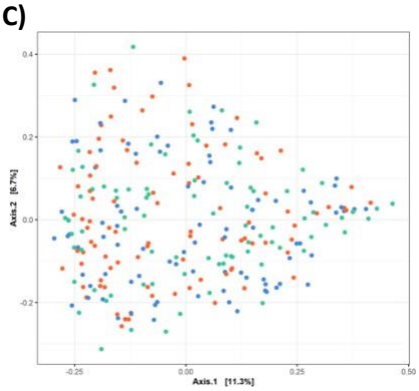
A)



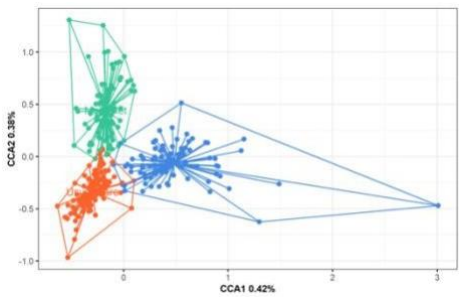
B)



C)



D)



E)

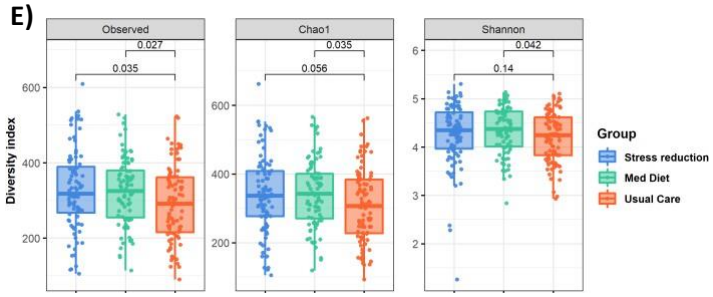


Figure 2.

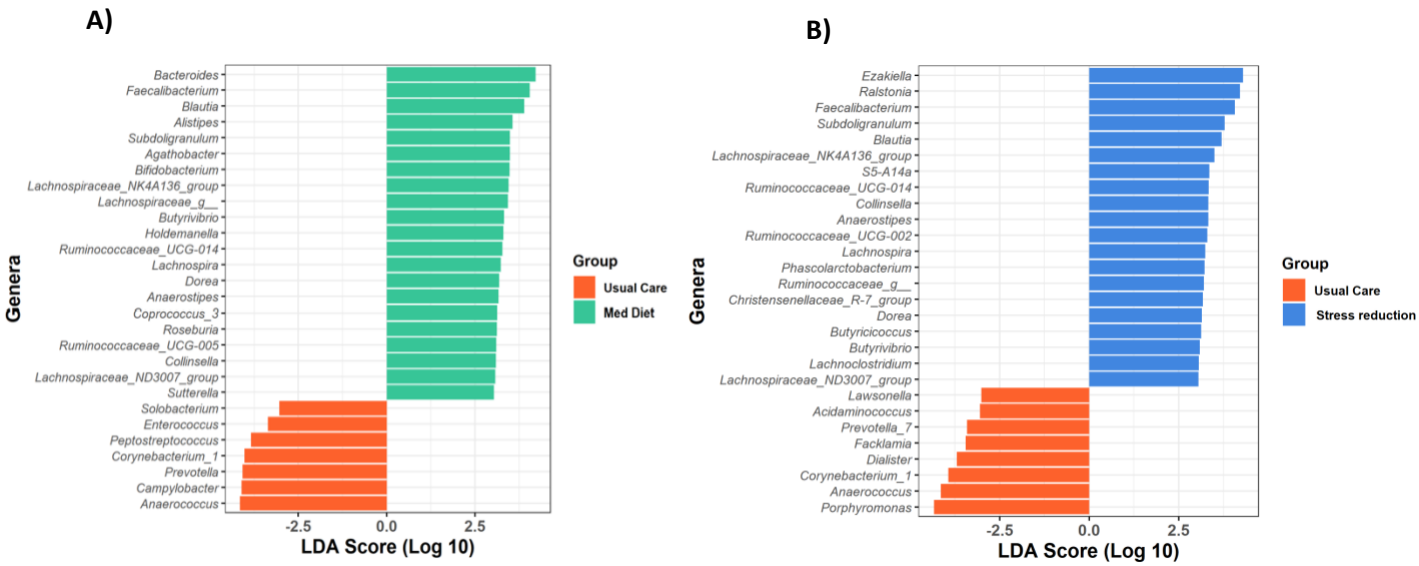
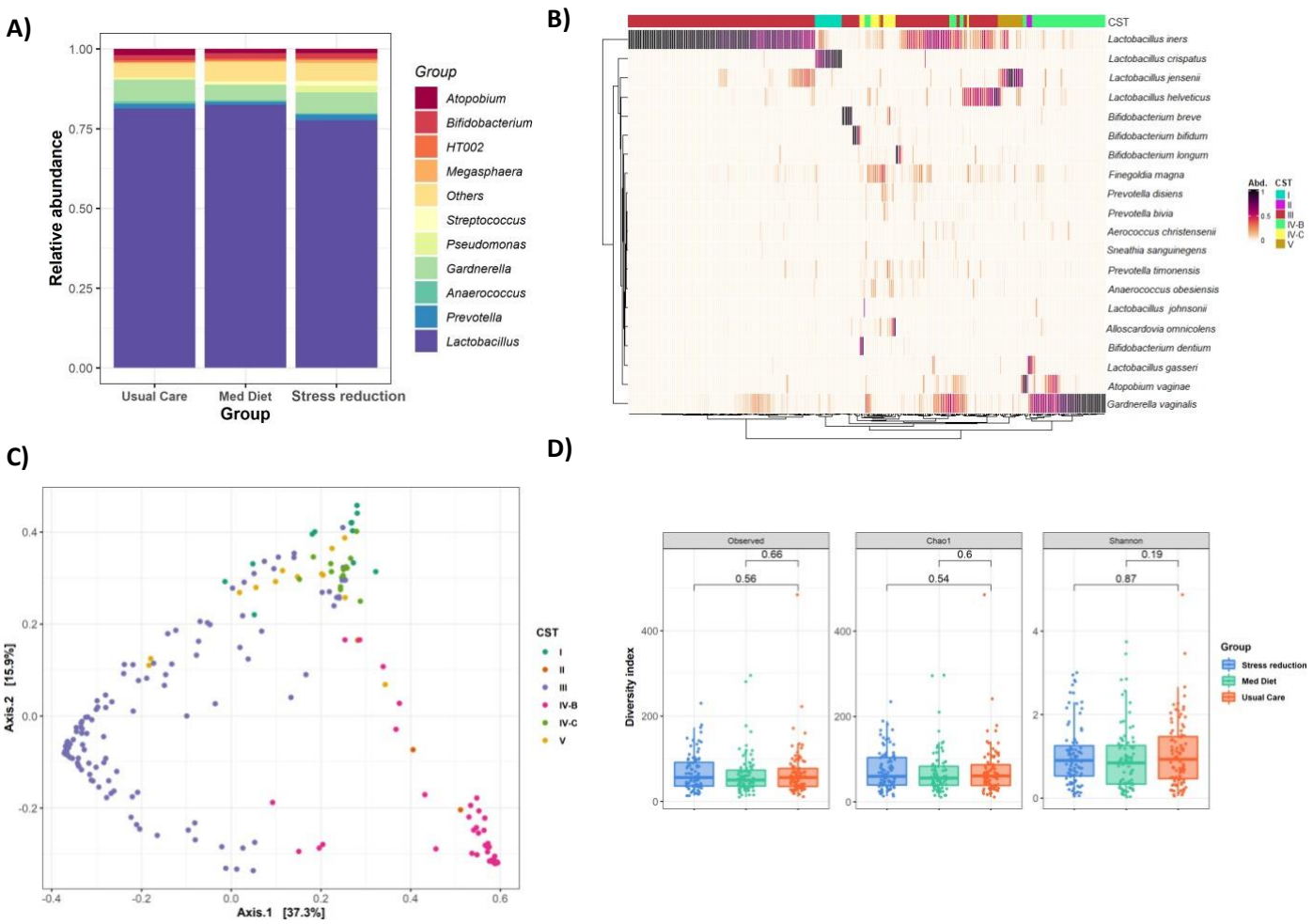

A)
Bacteroides
Faecalibacterium
Blautia
Alistipes
Subdoligranulum
Agathobacter
Bifidobacterium
Lachnospiraceae_NK4A136_group
Lachnospiraceae_g__
Butyrivibrio
Holdemanella
Ruminococcaceae_UCG-014
Lachnospira
Dorea
Anaerostipes
Coprococcus_3
Roseburia
Ruminococcaceae_UCG-005
Collinsella
Lachnospiraceae_ND3007_group
Sutterella
Solobacterium
Enterococcus
Peptostreptococcus
Corynebacterium_1
Prevotella
Campylobacter
Anaerococcus
Genera
-2.5
0.0
2.5
LDA Score (Log 10)
Group
Usual Care
Med Diet
B)
Ezakiella
Ralstonia
Faecalibacterium
Subdoligranulum
Blautia
Lachnospiraceae_NK4A136_group
S5-A14a
Ruminococcaceae_UCG-014
Collinsella
Anaerostipes
Ruminococcaceae_UCG-002
Lachnospira
Phascolarctobacterium
Ruminococcaceae_g__
Christensenellaceae_R-7_group
Dorea
Butyricicoccus
Butyrivibrio
Lachnoclostridium
Lachnospiraceae_ND3007_group
Lawsonella
Acidaminococcus
Prevotella_7
Facklamia
Dialister
Corynebacterium_1
Anaerococcus
Porphyromonas
Genera
-2.5
0.0
2.5
LDA Score (Log 10)
Group
Usual Care
Stress reduction

Figure 3.


A)
Relative abundance
1.00
0.75
0.50
0.25
0.00
Usual Care
Med Diet
Stress reduction
Group
Group
Atopobium
Bifidobacterium
HT002
Megasphaera
Others
Streptococcus
Pseudomonas
Gardnerella
Anaerococcus
Prevotella
Lactobacillus
B)
CST
Lactobacillus iners
Lactobacillus crispatus
Lactobacillus jensenii
Lactobacillus helveticus
Bifidobacterium breve
Bifidobacterium bifidum
Bifidobacterium longum
Finegoldia magna
Prevotella disiens
Prevotella bivia
Aerococcus christensenii
Sneathia sanguinegens
Prevotella timonensis
Anaerococcus obesiensis
Lactobacillus johnsonii
Alloscardovia omnicolens
Bifidobacterium dentium
Lactobacillus gasseri
Atopobium vaginae
Gardnerella vaginalis
Abd.
CST
I
II
III
IV-B
IV-C
V
C)
Axis.2 [15.9%]
Axis.1 [37.3%]
CST
I
II
III
IV-B
IV-C
V
D)
Observed
Chao1
Shannon
0.66
0.56
0.6
0.54
0.19
0.87
Diversity index
Group
Stress reduction
Med Diet
Usual Care

SUPLEMENTARY DATA

Materials and Methods

The effect of the gestational intervention on the maternal microbiota was conducted using RStudio through several packages, mainly phyloseq[1], vegan[2] and microbiomeMarker[3] packages. Alpha-diversity index both microbial richness and diversity was assessed by the calculation of Observed and Chao1 index and Shannon index, respectively by the phyloseq package. These indexes were calculated on the rarefied data to the minimum sample size for fecal microbiota (reads=24443) and vaginal (reads=34811) datasets. Rarefaction threshold was set considering those samples with number of reads higher than 8000 (n=3 samples were not included for alpha diversity analysis in the fecal dataset).

Differences in beta diversity of the microbial communities were evaluated by the Permutational Multivariate Analysis of Variance based on the Bray-Curtis distance using adonis2 function from the vegan package at Amplicon Sequence Variant (ASV) level. Differences in the overall structure of the microbial community was visualized using Principal Coordinate analysis (PCoA) plot using Bray-Curtis distance and the Canonical Coordinate analysis (CCA) plots using phyloseq, vegan and ggordiplot package[4(p1)]. Additionally, principal components analysis (PCA) on the centered-log ratio transformed (CLR) data at genus level was also performed to visualize the distribution of the samples according microbiota composition using FactoMiner and Factoextra packages[5,6].

Differential abundance analysis in microbiota composition was conducted using microbiomeMarker package[3] through the Linear discriminant analysis Effect Size analysis and Kruskal-Wallis test on the CLR transformed data. Furthermore, the online

platform microbiomeanalyst was also used to test differences in microbiota composition between conditions[7]. A size-effect cut-off of 3.0 on the logarithmic LDA score and q-value<0.2 were set as a significance threshold. Multivariate analyses were performed using the adjusted general linear models using Maaslin2 R package[7]. All multiple comparisons were adjusted using False Discovery rate and FDR adjusted p-value is referred in the manuscript as q-value (q-value <0.2 is considered for statistical significance). VALENCIA (VAginaL community state typE Nearest CentroId clAssifier) tool was used to assess the classification of the mothers based on their community state type (CST) from their vaginal microbiota composition[8]. All graphs were plotted using the following packages: ggplot2[9], ggpubr[10], forcats[11].

Results

Association with Mediterranean diet nutrients

In order to assess the potential association of this microbial taxa with different nutrients that define Mediterranean diet, we assessed the correlation between the consumption of specific foods and the taxa from maternal gut microbiota. When specific aliments were considered, some patterns were found related to groups of microbial taxa (**Figure S2**). Thus, food typically included in the Mediterranean diet such as legumes and blue fish showed a trend to positive correlation with the relative abundance of marker families of the Mediterranean diet intervention including *Ruminococcaceae*, *Lachnospiraceae* and *Bacteroidaceae* families. On the contrary, *Campylobacteriaceae* family showed a positive association with refined flour (rho=0.156, *p=0.035*) while

negative with integral flour (rho=-0.135, *p=0.035*), fiber (rho=-0.111, *p=0.084*) and fruits (rho=-0121, *p=0.061*) consumption (**Figure S2**).

Association with stress-related questionnaires

The potential association of those overrepresented marker taxa with the WHO-5 Well-Being Index Questionnaire was assessed in the whole population. Mothers were divided in two groups according to their WHO well-being indicator and differences in gut microbial taxa between them were assessed. Those mothers considered with a low well-being score (WHO-5 $\leq$52) showed an enrichment in Epsilonbacteraeota phylum (*p=0.001*, *q=0.017*) mainly due to the higher relative abundance of Campylobacteriaceae (*p=0.001*, *q=0.062*) family (**Table SIV**). Moreover, we found positive association between the WHO well-being score with *Faecalibacterium* (rho=0.124, *p=0.047*) genus and some genera from *Lachnospiraceae* family including *Lachnospiraceae*_FCS020_group (rho=0.129, *p=0.026*) and *Lachnospiraceae*_NK4A136_group (rho=0.131, *p=0.036*) (**Table SV)**.

Table S I. Maternal consumption of key dietary food intake at baseline and final according to intervention groups.

		Within-group mean changes				Between-group changes		
		Usual care	**Mediterranean diet**	**Stress reduction**		**MedDiet *vs.* Usual care**	**Stress reduction *vs.* Usual care**	**MedDiet *vs.* Stress reduction**
		n=92	n=80	n=88	*P*§	*Difference (95% CI)*	*Difference (95% CI)*	*Difference (95% CI)*
Extra-virgin Olive Oil - g/d	Baseline†	27.8 ± 19.0	32.2 ± 19.1	32.1 ± 19.4				
	Final‡	31.4 ± 1.8	40.5 ± 1.9**	31.5 ± 1.8	0.001	9.0 (3.8 to 14.3)	0.1 (-5.0 to 5.2)	9.0 (3.7 to 14.2)
Refined olive oil - g/d	Baseline†	8.6 ± 16.7	9.2 ± 16.1	8.0 ± 13.7				
	Final‡	5.8 ± 1.3	1.8 ± 1.4**	8.3 ± 1.3	0.003	-4.0 (-10.2 to -2.7)	2.4 (-1.2 to 6.1)	-6.5 (-10.2 to -2.7)
Total nuts - g/d	Baseline†	16.1 ± 17.9	18.9 ± 19.7	18.8 ± 21.7				
	Final‡	15.8 ± 1.8	32.9 ± 2.0**	17.0 ± 1.9	<0.001	17.0 (11.7 to 22.3)	1.1 (-4.0 to 6.2)	16.0 (10.6 to 21.3)
Vegetables - g/d	Baseline†	287.4 ± 140.1	291.6 ± 141.3	299.6 ± 136.9				
	Final‡	275.1 ± 10.0	343.2 ± 10.8**	269.4 ± 10.2*	<0.001	68.1 (39.1 to 97.1)	-5.7 (-33.8 to 22.3)	73.8 (44.7 to 103.0)
Legumes (g/d)	Baseline†	49.4 ± 38.8	50.2 ± 34.5	46.8 ± 39.0				
	Final‡	50.6 ± 4.0	79.3 ± 4.3**	44.8 ± 4.1	<0.001	28.7 (17.2 to 40.2)	-5.8 (-17.0 to 5.4)	34.5 (22.9 to 46.1)
Fruits (g/d)	Baseline†	304.2 ± 172.1	323.6 ± 155.0	333.4 ± 187.2				
	Final‡	347.4 ± 21.8*	416.6 ± 23.3**	331.9 ± 21.8	0.020	69.2 (6.7 to 131.7)	-15.5 (-76.0 to 45.0)	84.7 (22.3 to 147.2)
Refined cereals (g/d)	Baseline†	72.5 ± 44.6	62.0 ± 40.8	66.4 ± 54.4				
	Final‡	63.5 ± 4.1	33.6 ± 4.4**	62.1 ± 4.2	<0.001	-29.9 (-41.8 to -18.0)	-1.4 (-13.0 to 10.1)	-28.4 (-40.4 to -16.5)
Whole grain cereals (g/d)	Baseline†	37.7 ± 40.5	39.2 ± 44.5	42.8 ± 49.2				
	Final‡	36.9 ± 3.6	63.3 ± 3.9**	40.4 ± 3.7	<0.001	26.4 (16.1 to 36.7)	3.5 (-6.6 to 13.6)	-22.9 (-33.4 to -12.5)
Fish or seafood (g/d)	Baseline†	69.7 ± 39.6	75.1 ± 43.1	67.9 ± 37.8				
	Final‡	73.0 ± 4.3	101.6 ± 4.7**	74.7 ± 4.4	<0.001	28.5 (16.1 to 41.0)	1.7 (-10.4 to 13.8)	26.8 (14.2 to 39.5)
Fat fish (g/d)	Baseline†	13.0 ± 15.8	13.6 ± 14.9	15.0 ± 17.1				
	Final‡	13.3 ± 1.9	32.2 ± 2.0**	17.5 ± 1.9	<0.001	18.9 (13.5 to 24.2)	4.2 (-1.0 to 9.4)	14.7 (9.3 to 20.1)
Lean meat (g/d)	Baseline†	66.0 ± 38.7	79.4 ± 39.1	61.1 ± 34.7				
	Final‡	67.0 ± 3.4	79.3 ± 3.7	68.5 ± 3.5	0.035	12.2 (2.3 to 22.2)	1.5 (-8.1 to 11.0)	10.8 (0.7 to 20.9)
Red meat (g/d)	Baseline†	49.1 ± 38.3	55.4 ± 38.7	47.5 ± 29.1				
	Final‡	50.1 ± 3.2	39.4 ± 3.4**	50.5 ± 3.2	0.029	-10.7 (-19.8 to -1.6)	0.3 (-8.5 to 9.2)	-11.1 (-20.3 to -1.8)
Processed meat (g/d)	Baseline†	35.4 ± 26.9	32.7 ± 23.0	43.2 ± 45.0				
	Final‡	31.1 ± 2.4*	37.6 ± 2.4	33.2 ± 2.3*	0.145	6.7 (-0.2 to 13.6)	1.6 (-5.1 to 8.3)	5.1 (-1.9 to 12.1)
Pastries. cakes or sweets (g/d)	Baseline†	46.5 ± 35.4	42.4 ± 43.4	33.7 ± 31.9				
	Final‡	41.0 ± 3.1	28.8 ± 3.3*	39.5 ± 3.1	0.015	-12.2 (-21.1 to -3.2)	-1.5 (-10.2 to 7.3)	-10.7 (-19.7 to -1.7)
Dairy products (g/d)	Baseline†	341.8 ± 225.1	359.6 ± 246.5	376.5 ± 237.9				
	Final‡	334.3 ± 19.0	468.5 ± 20.2**	353.9 ± 19.5	<0.001	134.2 (79.8 to 188.6)	19.7 (-33.8 to 73.2)	114.5 (59.5 to 169.6)
MedDiet Score	Baseline†	7.0 ± 2.6	7.8 ± 2.4	7.5 ± 2.7				
	Final‡	7.5 ± 0.2	12.2 ± 0.2**	7.8 ± 0.2	<0.001	4.6 (4.0 to 5.3)	0.3 (-0.4 to 0.9)	4.4 (3.7 to 5.0)

MedDiet: Mediterranean diet; EVOO: Extra Virgin Olive Oil.

†Baseline values are observed means ± SD. ‡ Final values are baseline-adjusted (least-squares) means ± SE and comparison among groups done with ANCOVA analysis.

*P<0.05 and **P<0.001 final from baseline comparison. § ANCOVA analysis.

Data available for 260 pregnancies (N=92 Usual care, N=80 Mediterranean diet, N=88 Stress reduction).

Table S II. Maternal dietary energy and nutrient intake at baseline and final according to intervention groups.

		Within-group mean changes				Between-group changes		
		Usual care n=85	**Mediterranean diet** n=74	**Stress reduction** n=83	*P*§	**MedDiet *vs.* Usual care** *Difference*	**Stress reduction *vs.* Usual care** *Difference*	**MedDiet *vs.* Stress reduction** *Difference*
Energy - kcal/d	Baseline†	2493.0 ± 495.6	2506.9 ± 581.6	2441.8 ± 519.0				
	Final‡	2410.6 ±48.3	2611.0 ± 51.7	2403.6 ± 48.9	<0.001	200.4 (61.8 to 339.1)	5.6 (-121.8 to 133.1)	207.4 (67.9 to 347.0)
Protein - g/d	Baseline†	104.0 ± 23.5	108.9 ± 26.8	104.3 ± 25.1				
	Final‡	101.4 ± 2.4	120.4 ± 2.6**	103.0 ± 2.4	<0.001	19.0 (12.1 to 25.9)	1.58 (-5.1 to 8.3)	17.4 (10.4 to 24.4)
Carbohydrate - g/d	Baseline†	234.3 ± 61.3	224.3 ± 66.0	219.6± 64.8				
	Final‡	219.7 ± 5.3	218.8 ± 5.6	212.4 ± 5.3	0.575	-0.9 (-16.0 to 14.2)	-7.3 (-22.0 to 14.2)	6.4 (-8.8 to 21.6)
Fiber - g/d	Baseline†	32.8 ± 12.0	32.6 ± 9.3	32.5 ± 11.6				
	Final‡	32.0 ± 0.9	38.1 ± 1.0**	30.9 ± 1.0	<0.001	6.1 (3.3 to 8.8)	-1.1 (-3.8 to 1.5)	7.2 (4.4 to 9.9)
Total fat - g/d	Baseline†	132.3 ± 30.5	135.8 ± 35.8	134.0 ± 36.8				
	Final‡	130.5 ± 3.2	147.4 ± 3.5**	132.7 ± 3.3	0.001	17.0 (7.6 to 26.3)	2.2 (-6.8 to 11.3)	14.7 (5.4 to 24.1)
SFA - g/d	Baseline†	36.2 ± 9.1	37.2 ± 12.0	36.3 ± 9.7				
	Final‡	35.5 ± 0.9	34.4 ± 1.0	35.7 ± 0.9	0.743	1.0 (-1.6 to 3.5)	0.2 (-2.3 to 2.7)	0.7 (-1.8 to 3.3)
MUFA - g/d	Baseline†	64.5 ± 16.7	67.5 ± 18.4	67.0 ± 21.2				
	Final‡	64.9 ± 1.8	72.4 ± 1.9*	67.0 ± 1.8	0.014	7.5 (2.4 to 12.7)	2.1 (-2.9 to 7.1)	5.4 (0.25 to 10.6)
PUFA - g/d	Baseline†	20.7 ± 8.0	19.6 ± 6.8	19.2 ± 6.8				
	Final‡	19.3 ± 0.7	25.5 ± 0.8**	18.8 ± 0.7	<0.001	6.1 (4.0 to 8.3)	-0.6 (-2.6 to 1.5)	6.7 (4.6 to 8.8)
α-Linoleic acid - g/d	Baseline†	15.6 ± 7.0	14.4 ± 5.5	13.8 ± 5.5				
	Final‡	14.4 ± 0.6	18.0 ± 0.7**	13.7 ± 0.6	<0.001	3.6 (1.8 to 5.4)	-0.7 (-2.4 to 1.07)	4.2 (2.5 to 6.0)
α-Linolenic acid - g/d	Baseline	1.4 ± 0.6	1.5 ± 0.6	1.4 ± 0.7				
	Final‡	1.2 ± 0.1*	2.3 ± 0.1**	1.3 ± 0.1	<0.001	1.1 (0.9 to 1.2)	0.1 (-0.1 to 0.2)	1.0 (0.8 to 1.2)
EPA - g/d	Baseline†	0.1 ± 0.1	0.2 ± 0.1	0.15 ± 0.1				

	Final‡	0.2 ± 0.01	0.3 ± 0.01**	0.2 ± 0.01	<0.001	0.1 (0.08 to 0.14)	0 (-0.01 to 0.05)	0.1 (0.06 to 0.12)
DHA - g/d	Baseline†	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.3				
	Final‡	0.3 ± 0.03	0.6 ± 0.03**	0.4 ± 0.03	<0.001	0.3 (0.2 to 0.35)	0.05 (-0.02 to 0.13)	0.2 (0.14 to 0.3)
Trans-FA - g/d	Baseline†	1.8 ± 1.1	2.0 ± 1.5	1.5 ± 1.0				
	Final‡	1.8 ± 0.1	1.3 ± 0.1**	1.6 ± 0.1	0.003	-0.5 (-0.8 to -0.2)	-0.2 (-0.5 to 0.08)	-0.3 (-0.6 to 0)
Cholesterol - mg/d	Baseline†	343.9 ± 90.8	364.9 ± 128.0	333.2 ± 93.3				
	Final‡	338.4 ± 10.3	358.0 ± 11.0	342.4 ± 10.4	0.397	19.6 (-9.9 to 49.2)	4.0 (-24.6 to 32.6)	15.6 (-14.2 to 45.5)

MedDiet denotes for Mediterranean diet. MBSR Mindfulness-Based Stress Reduction. SFA Saturated fatty acids. MUFA Monounsaturated fatty acids. PUFA Polyunsaturated fatty acids. EPA Eicosapentaenoic acid. DHA Docosahexaenoic acid and FA Fatty acids.
†Baseline values are observed means ± SD. ‡ Final values are baseline-adjusted (least-squares) means ± SE and comparison among groups done with ANCOVA analysis. *P<0.05 and **P<0.001 final from baseline comparison. § ANCOVA analysis.

Data available for 242 pregnancies (N=85 Usual care, N=74 Mediterranean diet, N=83 Stress reduction).

Table S III. Maternal anxiety and wellbeing evaluated by validated questionnaires at baseline and final according to intervention groups.

		Within-group mean changes				Between-group changes		
		Usual care n=92	**Mediterranean diet** n=76	**Stress reduction** n=88	*P*§	**MedDiet *vs.* Usual care** *Difference (95% CI)*	**Stress reduction *vs.* Usual care** *Difference (95% CI)*	**Stress reduction *vs.* MedDiet** *Difference (95% CI)*
Perceived stress scale score	Baseline†	16.2 ± 7.4	16.7 ± 8.0	16.8 ± 7.6				
	Final‡	16.2 ± 0.7	16.0 ± 0.8	16.7 ± 0.7	0.786	-0.44 (-2.09 to 1.10)	0.13 (-1.48 to 1.74)	0.57 (-1.10 to 2.24)
State-trait Anxiety Inventory (anxiety)	Baseline†	14.6 ± 9.4	13.5 ± 9.1	14.5 ± 8.9				
	Final‡	15.9 ± 0.9	13.6 ± 0.9	13.4 ± 0.8	0.029	-1.64 (-3.52 to 0.24)	-2.42 (-4.24 to -0.60)	-0.78 (-2.68 to 1.12)
State-trait Anxiety Inventory (personality)	Baseline†	16.4 ± 9.2	14.3 ± 8.4	15.4 ± 8.2				
	Final‡	15.9 ± 0.8	14.8 ± 1.0	14.2 ± 0.8	0.247	0.46 (-1.18 to 2.09)	-0.91 (-2.48 to 0.67)	-1.37 (-3.01 to 0.28)
Five well-being index	Baseline†	61.6 ± 17.6	68.7 ± 14.7	65.6 ± 17.2				
	Final‡	62.7 ± 1.8	66.5 ± 1.7	67.1 ± 1.8	0.484	-0.35 (-4.51 to 3.80)	1.97 (-2.03 to 5.97)	2.32 (-1.83 to 6.47)
FFMQ: Observation	Baseline†	24.5 ± 5.9	24.5 ± 5.6	22.7 ± 5.6				
	Final‡	24.0 ± 0.7	24.9 ± 0.7	27.3 ± 0.6**	<0.001	0.00 (-1.43 to 1.42)	3.50 (2.13 to 4.87)	3.51 (2.06 to 4.95)
FFMQ 2: Description	Baseline†	32.5 ± 5.2	32.6 ± 5.1	31.8 ± 5.2				
	Final‡	31.8 ± 0.6	32.5 ± 0.5	32.3 ± 0.5	0.673	0.16 (-1.05 to 1.37)	0.52 (-0684 to 1.69)	0.36 (-0.87 to 1.58)
FFMQ 3: Awareness	Baseline†	31 .1 ± 6.3	31.6 ± 5.4	29.8 ± 6.7				
	Final‡	30.1 ± 0.7	30.6 ± 0.8	29.8 ± 0.6	0.952	0.18 (-1.52 to 1.87)	0.26 (-1.38 to 1.90)	-0.08 (-1.65 to 1.81)
FFMQ 4: Non-judgmental	Baseline†	29.5 ± 6.0	30.1 ± 5.3	29.3 ± 5.9				
	Final‡	29.0 ± 0.6	29.9 ± 0.6	30.9 ± 0.5*	0.006	0.50 (-0.81 to 1.81)	1.98 (0.72 to 3.25)	1.48 (0.15 to 2.81)
FFMQ 5: Non-reactivity	Baseline†	22.4 ± 4.9	23.1 ± 5.6	21.7 ± 4.9				
	Final‡	22.9 ± 0.5	22.8 ± 0.6	24.2 ± 0.4**	0.002	-0.51 (-1.75 to 0.72)	1.65 (0.45 to 2.84)	2.16 (0.90 to 3.42)

MedDiet: Mediterranean diet; FFMQ Five Facet Mindfulness Questionnaire.
†Baseline values are observed means ± SD. ‡ Final values are baseline-adjusted (least-squares) means ± SE and comparison among groups done with ANCOVA analysis.
*P<0.05 and **P<0.001 final from baseline comparison. § ANCOVA analysis.

Data available for 256 pregnancies (N=92 Usual care, N=76 Mediterranean diet, N=88 Stress reduction).

Table S IV. Differences in gut microbiota composition according to their WHO-5 Well-Being Index.

	High well-being (WHO-5 score ≥52)	Low well-being (WHO-5 score <52)	p-value	q (FDR)
Phylum				
Epsilonbacteraeota	0.99 [0.39-2.37]	1.58 [0.843-2.6]	***0.001***	***0.017***
Proteobacteria	1.17 [0.43-1.85]	0.95 [0.31-1.52]	*0.141*	*0.896*
Verrucomicrobia	0.04 [0-0.41]	0.02 [0-0.12]	*0.281*	*0.896*
Family				
Campylobacteraceae	0.99 [0.39-2.37]	1.58 [0.85-2.61]	***0.001***	***0.062***
Aerococcaceae	0.05 [0-0.4]	0.13 [0.05-0.67]	*0.013*	*0.302*
Family_XI	29.22 [17.92-41.22]	33.94 [20.26-45.42]	*0.040*	*0.628*
Tannerellaceae	0.47 [0.12-0.86]	0.2 [0.03-0.73]	*0.069*	*0.666*
Veillonellaceae	2.29 [1.2-3.37]	2.335 [1.17-3.93]	*0.071*	*0.666*
Bacteroidaceae	3.59 [0.71-8.64]	2.36 [0.26-7.21]	*0.100*	*0.713*
Burkholderiaceae	0.33 [0.13-0.77]	0.26 [0.08-0.46]	*0.106*	*0.713*
Erysipelotrichaceae	0.68 [0.28-1.87]	0.52 [0.12-0.87]	*0.34*	*0.742*
Actinomycetaceae	0.74 [0.31-1.32]	1.01 [0.42-1.73]	*0.144*	*0.753*
Porphyromonadaceae	3.47 [0.95-6.86]	4.52 [1.56-9.74]	*0.167*	*0.784*
Genus				
Campylobacter	1.01 [0.4-2.4]	1.65 [0.865-2.69]	***0.001***	***0.202***
Bilophila	0.06 [0.02-0.13]	0.1 [0.05-0.65]	*0.019*	*0.864*
Facklamia	0.05 [0-0.38]	1.69 [0.94-2.75]	*0.020*	*0.864*
Aerococcus	0 [0-0]	0.03 [0-0.08]	*0.035*	*0.864*
Paraprevotella	0 [0-0.25]	0 [0-0.02]	*0.060*	*0.864*
Blautia	1.81 [0.49-4]	0 [0-0.06]	*0.061*	*0.864*

Mothers were classified according to their score after the WHO-5 Well-Being Index Questionnaire as a measure of their wellbeing score. Those mothers with a WHO-5< 52 was classified as a "low well-being score" and the rest are group as "high well-being score". Data is expressed as median [interquartile range]. Differences were assessed independently for each taxonomical level on the log-centered data using Mann-Whitney test on the Microbiomeanalyst web platform. Only those phyla and families with a p-value <0.3 and those genus with a p-value <0.7 are shown.

Table S V. Spearman correlation between pregnant women gut microbiota taxa at phylum and genus level and WHO-5 Well-Being score.

	Spearman's rho	p-value
Phylum		
Epsilonbacteraeota	-0.123	0.049
Verrucomicrobia	0.108	0.084
Proteobacteria	0.102	0.101
Synergistetes	-0.068	0.276
Actinobacteria	-0.063	0.313
Bacteroidetes	0.044	0.48
Firmicutes	0.027	0.668
Fusobacteria	-0.005	0.935
Genera		
Bilophila	0.189	0.002
Aerococcus	-0.184	0.003
Odoribacter	0.169	0.007
Parabacteroides	0.164	0.008
Erysipelotrichaceae_UCG-003	0.157	0.012
Butyricicoccus	0.144	0.021
Facklamia	-0.141	0.024
Marvinbryantia	0.14	0.024
Alistipes	0.14	0.025
Lachnospiraceae_FCS020_group	0.139	0.026
Phascolarctobacterium	0.139	0.026
Paraprevotella	0.138	0.026
Ruminococcaceae_NK4A214_group	0.136	0.029
Allisonella	0.133	0.033
Lachnospiraceae_NK4A136_group	0.131	0.036
Adlercreutzia	0.13	0.037
Senegalimassilia	0.13	0.037
Bacteroides	0.129	0.038
Coprococcus_2	0.127	0.042
Nosocomiicoccus	-0.124	0.047
CAG-56	0.124	0.047
Faecalibacterium	0.124	0.047
Actinomyces	-0.123	0.049

All the phyla are shown while only those genera that showed a correlation with a p-value <0.05 are shown in the table.

FIGURES

Figure S1. Distribution of the fecal microbiota profile according to intervention group and its associated genera. **A)** Biplot of the principal component analysis of the fecal microbiota at genus level and the most relevant genera that contribute to each component distribution. Data was transformed using centered-log ratio and only those variables with a contribution higher than 20 was included in the plot. **B)** Boxplot showing the distribution of the samples on the PCA1 component based on the composition of the fecal microbiota. Statistical difference between component value between groups was assessed by Wilcoxon rank-sum test.

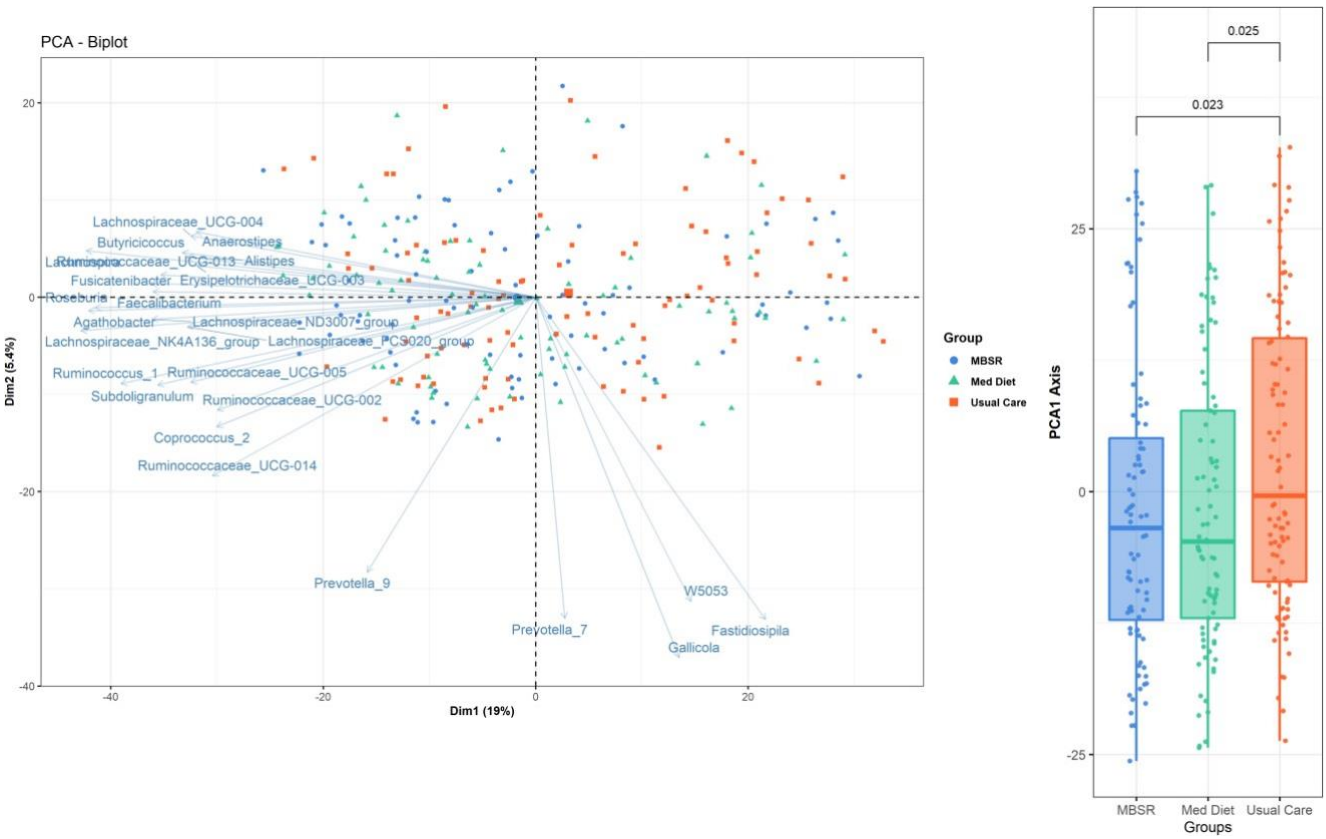
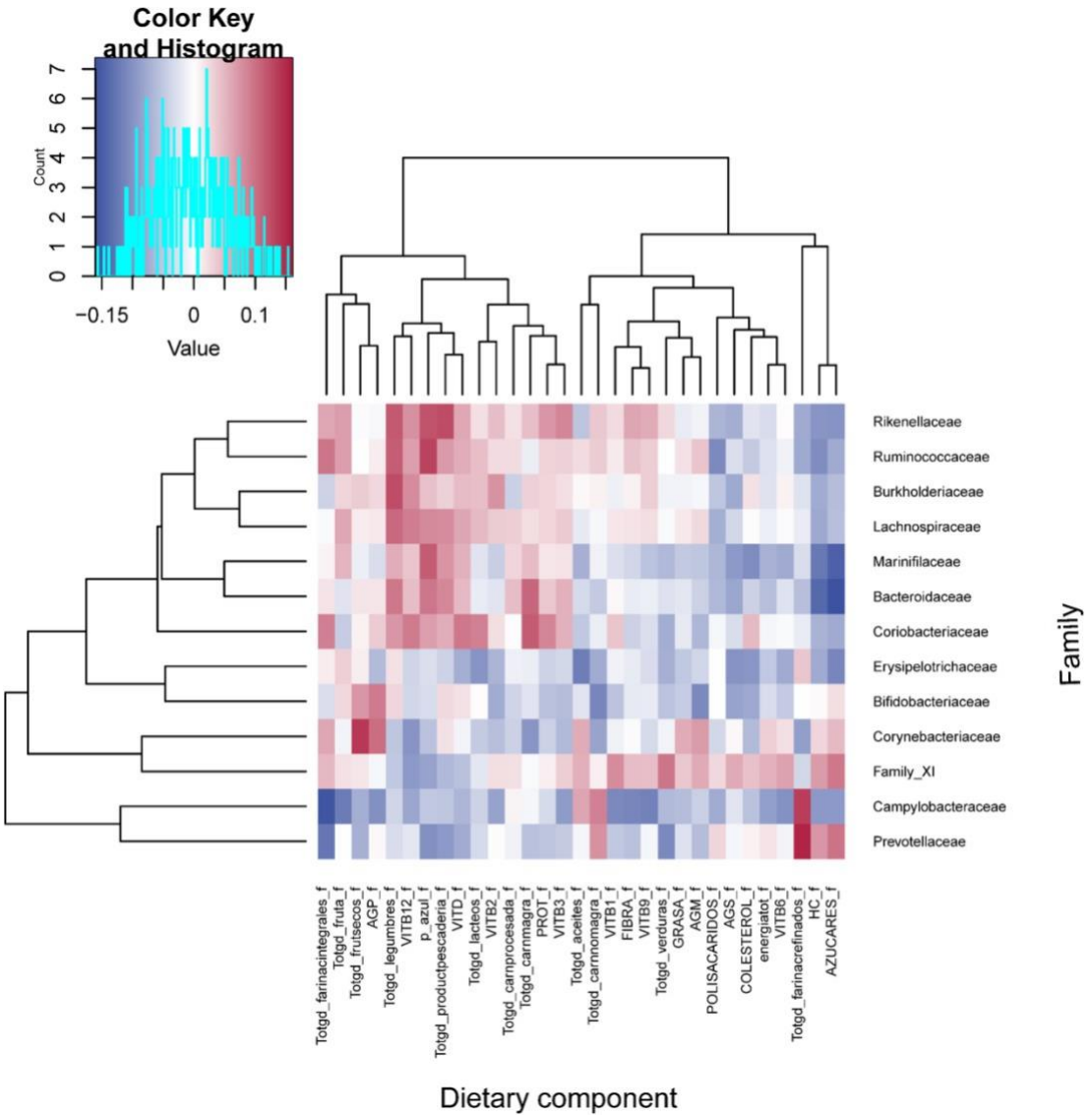


Figure S2. Heatmap of the Spearman's correlation between analyzed dietary components and selected taxa from pregnant women gut microbiota at family level. Those taxa highlighted in the Linear discriminant analysis Effect Size analysis as affected by the Mediterranean diet intervention and marked the difference between groups were included in the correlation analysis.



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7. Discussion

This thesis presents a total of 5 original studies in maternal mental health during pregnancy and the maternal microbiota.

7.1. Perceived Stress, Anxiety, Well-being and Sleep Disturbances during pregnancy

The high prevalence of antenatal negative affective states in a low-risk pregnant population is put into evidence in our studies: stress, anxiety, compromised well-being, and sleep disorders were reported by a significant number of pregnant participants in our cohort. Thus, nearly one quarter of participants scored as high stress and anxiety in the third trimester of pregnancy. Our results go in line with previous published evidence by Rondó *et al.*, who found high stress in between 22.1 and 24.6% of pregnant women in the three trimesters of pregnancy(12) and Dennis *et al.* who reported a prevalence rate for self-report anxiety symptoms of 24.6% in the third trimester.

Previous published studies had already shown that anxiety and depressive symptoms are not homogeneous during the perinatal period(49,136,137). In our cohort, perceived stress and STAI-T did not change throughout pregnancy; however, we did see that STAI-S increased in the third trimester of pregnancy.

The percentages of perceived stress and anxiety found in our cohort are very similar to those expected according to previous literature, and they put into manifest the importance of targeting these patients with clinically validated questionnaires in routinary pregnancy

follow-up, with the aim to offer supported interventions. Moreover, previous evidence has suggested that pregnancy-related anxiety constitutes a different concept than general anxiety(138). This fact could be a possible explanation for a limited measurement and assessment of anxiety in pregnancies and could also encourage the need of research in pregnancy-adapted measurement tools(138). Future research is needed in this topic.

Around 26% of our population had a compromised well-being. To the best of our knowledge, at the time of writing this thesis, there is no published data to compare with our results, as far as the prevalence of compromised well-being in general pregnancy population is concerned. However, in a study conducted by Sattler *et al.* in a group of overweight and obese women in Europe, they report a prevalence of low well-being of 27% before 20 weeks of pregnancy(139). Similarly, Mortazavi *et al.* during the Covid-19 pandemic reported a prevalence of compromised well-being of 24.4% pregnant women during gestation(6). These percentages are similar to the results we found.

The WHO-5 questionnaire is considered a good screening questionnaire with high sensitivity and specificity for clinical depression(139). It has the advantage of being self-administered, relatively easy and quick to use in daily clinical practice allowing a first detection of women with a negative affective state that could benefit from a further mental health assessment.

The prevalence of sleep disturbances in our cohort was very high: more than 80% of participants were found to have compromised sleep quality at 34-36 weeks of gestation. Our prevalence results are higher than expected according to the literature, ranging between 17 and 76%(14). As suggested in previous studies, this fact could put into manifest the possibility that validated cut-off for sleep questionnaires in general population may not be valid in pregnancies, the latter requiring a higher score(15).

Moreover, we found that sleep quality questionnaires worsened in the third trimester assessment compared to the results found previously in pregnancy. The worsening of sleep quality throughout gestation found in our cohort is in line with previous evidence: according to a metanalysis of 24 studies where they found that sleep disturbances tend to increase during pregnancy and clinicians should be aware that complaints of very poor sleep could require intervention(15).

Diagnosis and screening of maternal mental health is recommended by scientific societies using a standardized and validated tool(53). Our results stress this recommendation. However, we are certain that the use of multiple questionnaires to assess maternal mental health and sleep quality can be challenging in daily clinical practice, especially in high healthcare workload. Therefore, we believe understanding the risk factors associated may help targeting those patients at higher risk, as they can be identified at the beginning of pregnancy. Moreover, when correctly identified, these patients have the potential opportunity to receive pharmacological or intervention treatment and thus facilitating daily clinical practice.

7.2. Risk factors for compromised maternal mental health during pregnancy

We were interested in understanding the potential risk factors for compromised maternal mental health. In our cohort, we found that a risk factor for maternal perceived stress, a higher state anxiety level and a poorer well-being at third trimester were non-university studies. In line with these results, some previous research in pregnant population had already postulated low educational profile as a risk factor for antenatal depression(48,49). However, in contrast to our findings, Lancaster *et al.*, in a systematic review described only a small

association of lower educational level with depressive symptoms, but it was not significantly associated in the multivariate analysis(50). In general non-pregnant population, low educational level has also been associated to anxiety and depression(140). Education is more likely to result in good mental health rather than result from good mental health, and it may generally provide success in pursuing personal ends that include emotional well-being(140,141), thus possibly explaining our results.

For STAI-T personality questionnaire, we found preeclampsia in a previous pregnancy to be a potential risk factor. In a systematic review, Grigoriadis *et al.* found that prenatal maternal anxiety was not significantly associated with preeclampsia, although there was a significant heterogeneity across studies(142). However, we did not find in previous literature, any data regarding the association between previous preeclampsia and compromised mental health in the following pregnancies. A previous history of obstetric adverse events has already been related to symptoms of anxiety and depression(143), which could go in line with our results regarding the occurrence of preeclampsia in a previous pregnancy.

Perceived stress was also influenced in our cohort by non-white ethnicity and maternal age <40 years, and the latter was also found to be a risk factor for a higher score in trait anxiety among our participants. The literature also provides inconsistent findings as far as maternal age and ethnicity are concerned, as reported in the systematic review by Lancaster *et al.*(50) and Biaggi *et al.*(49). In their review, Biaggi *et al.* described 13 studies where young age was postulated as a risk factor, in contrast with 10 studies where advanced maternal age was described as a risk factor for antenatal depression and anxiety(49).

A higher state anxiety level at third trimester and a poorer maternal well-being at third trimester assessment were provided by the presence of a previous psychiatric disorder. These

results go in line with previously published evidence, as previous mental health disorders have been strongly related to higher anxiety in the past, in particular a history of anxiety and depression and a history of psychiatric treatment(49,71,81,82,144)

As for poor maternal sleep quality, no significant contributing factors were found. These findings contrast with those found by previous research where some risk factors could be postulated as contributors to sleep disturbances during pregnancy such as stillbirth history, general health-related quality of life or insufficient physical activity(14). Christian *et al*. found that African-American ethnicity and multiparity were related to poor sleep during pregnancy(145). Other studies reported gestational age(15) or previous maternal Body Mass Index (BMI) to be contributing factors(146). Our univariate analysis also suggested ethnicity and obesity to be contributing factors, however we could not demonstrate it in the multivariate analysis.

Thus, when analyzing previous literature on risk factors for compromised maternal mental health, inconsistent findings have been found as described earlier on. With high probability, this fact is due to complex multifactorial etiology for this conditions(50), but also to a complex interpersonal variability to the reaction of a certain experience, which in the end may conditionate the final response to a new distressor and could be an explanation to the differences found in final pregnancy and offspring outcomes(52).

7.3. Effects of the maternal environment on maternal health during pregnancy

We also wanted to learn the potential effects on maternal mental health that **a negative maternal environment** could have. To do so, we evaluated the maternal mental status during the COVID-19 pandemic, which provided us a unique opportunity to study the effects of a

negative common external stressor for the population. The well-being score in almost half (43%) of our study population was low (less than 52 in the WHO-5 questionnaire), thus demonstrating that maternal well-being status during a negative maternal environment was affected.

Our results were in line with those expected from previous evidence. Literature from previous pandemics like H1N1 and SARS already postulated the negative psychological impact of mandatory quarantine in non-pregnant population(66). When studying pregnant population, concerns were raised on the even higher psychological impact for expecting mothers, due to, for example, disrupted prenatal care and delivery, with women being asked in many countries to attend to their prenatal visits alone or even to give birth alone, despite knowing that the familiar support during birthing process is considered to be essential(147).

Several studies reported a compromised maternal mental status during the COVID-19 pandemic(65,67–69). Prevalence of depressive and anxiety symptoms ranging around 15–19% and 11–31%, respectively, were found(67,68). Most of these works were based on maternal depression and anxiety scales. In contrast to our results, only a limited number used maternal well-being as an assessment of maternal physical, mental, and social health(6) to adequately compare with our study. The percentage of patients with low WHO-5 score reported by the authors was 24,4%, which is significantly lower to our results (43%).

In our study, we found that the negative psychological impact on the well-being of mothers during the COVID-19 pandemic, was even more evident when we compared pandemic versus pre-pandemic cohorts, where the 43% of compromised well-being scores contrasted with around 28% of the latter cohort.

We found only few studies comparing pandemic cohort data to a previous pre-pandemic cohort. During the COVID-19 pandemic, Wu *et al.* reported higher depression symptoms in comparison to a pre-pandemic cohort and found a positive association with the number of newly COVID-19 confirmed cases, suspected cases and deaths(70). Similarly, Berthelot *et al.* described women from their COVID-19 pandemic being more likely to present depressive and anxiety symptoms than their pre-pandemic cohort, especially when having a previous psychiatric diagnosis or low income(71). Perzow *et al.* compared 135 patients pre and post pandemic and found higher levels of anxiety and depression during the pandemic(72). Zanardo *et al*. reported higher scores for anhedonia and depression when comparing to 100 pre-pandemic patients(73). Interestingly, however, Dong *et al*. found that the anxiety levels of pregnant women was the same as before the pandemic, while the level of depression was significantly higher. They found no differences in terms of gestational age or testing positive for Sars-CoV-2 infection(77). To the best of our knowledge, ours was the first study that assesses maternal well-being before and after the pandemic.

In a situation of global pandemics, healthcare workers may be under unique clinical demands. Identifying patients with compromised mental status may be especially difficult under such circumstances. In this line, we again tried to identify potential risk factors that contributed to a worse well-being during pregnancy in the COVID-19 pandemic, with the aim to correctly identify patients at higher risk which could benefit from further mental assessment.

We found that previous psychiatric disease, being in the third trimester of pregnancy, and hospital admission for COVID-19 disease were risk factors for a negative maternal mental well-being. The infection of SARS-CoV-2 itself did not increase the risk of a lower well-being condition, but the severity of COVID-19 disease requiring hospitalization did. Similarly, some studies had reported that a previous psychiatric disorder diagnosed in pregnant women was

a risk factor for depression symptoms during the COVID-19 pandemic(71,81,82). The stage of pregnancy had a unique association with anxiety and the level of well-being. Zeng *et al.* reported that the third trimester of pregnancy at the time of the COVID-19 pandemic seemed to be associated with a worse maternal well-being, with even worse results in comparison to the post-partum period(67). On the contrary, Saccone *et al.* found worse results in anxiety and psychological impact in pregnant women in the first trimester(69). Other authors found no differences according to gestational age(75–77).

COVID-19 symptoms and infection had been described as anxiety risk factors(78) and predictors for post-traumatic stress disorder(79). However, these studies did not consider the differences between confirmed SARS-CoV-2 infected and healthy patients, like we did. In our study, we had the initial hypothesis that SARS-CoV-2 infection may increase the level of anxiety and worsen mental condition; however, our data did not confirm this hypothesis as found in other studies with smaller sample sizes(77,80). We did report worse maternal well-being in SARS-CoV-2 infected mothers with severe symptoms or requiring hospital admission due to COVID-19 disease for respiratory and or medical support according to our center protocols at the time of the study.

From an opposite point of view, we also wanted to know the effects on maternal mental health that **a positive maternal environment** could have. To do so, we present the results in the context of a randomized clinical trial involving pregnant women at high risk for an Small for Gestational Age (SGA) newborn (The IMPACT BCN Trial)(101). Under a positive maternal environment, such as an intervention based on Mediterranean diet, we found a significant reduction of maternal anxiety and stress and an improvement of maternal well-being and sleep quality.

There has been increasing interest on the effects of the diet on mental health, stress, and quality of life in general(86). Our results go in line with previous evidence on the beneficial effects of the Mediterranean diet found in general population: Jacka *et al.* conducted a randomized controlled trial to investigate the efficacy of a dietary intervention based on the Mediterranean diet for the treatment of symptoms in subjects with Major Depressive Disorder. The Mediterranean diet group showed significantly greater improvements in symptoms of depression compared to the control group(98). Additionally, in the PREDIMED study, a preventive effect (40% lower risk compared to the control arm) for depression was found for the Mediterranean diet group supplemented with nuts in participants with type 2 diabetes(99).

However, the evidence about the effects of dietary interventions on mental health during pregnancy is limited. Our study reveals that following the Mediterranean diet during pregnancy is associated with a reduction in maternal anxiety and stress in self-reported questionnaires. These findings are in line with previous data: Papandreou *et al.* conducted a randomized clinical trial with 40 pregnant women incorporating Mediterranean diet recommendations showing an improvement in nutritional status and reduction in health-related anxiety and depression(148). Similarly, Flor-Alemany *et al.* in a longitudinal study of 152 pregnant women, a secondary analysis of the GESTAFIT trial, showed that higher adherence to the Mediterranean diet was inversely associated with anxiety and directly associated with emotional regulation, resilience and positive affect in both second and third trimester of pregnancy(102). Moreover, these associations were significant whole-grain cereals, fruits and vegetables, extra-virgin olive oil and nuts, food sources of dietary antioxidants whose consumption was encouraged during the intervention in our study and considered key foods of the Mediterranean diet(102).

Our study also demonstrates an improvement in maternal well-being and sleep quality with Mediterranean diet. The association between higher Mediterranean diet adherence and subjective well-being has been found in observational studies(149). In our third study, we also described a positive association with maternal sleep quality and the Mediterranean diet intervention. Again, our results go in line with previous evidence in both pregnant(106) and non-pregnant population(104,105). However, to our knowledge, the present study is the first randomized clinical trial with a structured intervention based on a Mediterranean diet adapted to pregnancy to evaluate well-being and sleep quality.

Several biological mechanisms have been postulated regarding the relationship between diet and mental health. The Mediterranean diet is a dietary pattern which provides recommendations of proportion and frequency of consumption, but also promotes a healthy lifestyle, including cultural and lifestyle elements such as moderation, socialization, physical activity and also rest. It is not just about prioritizing some food groups from others, but also paying attention to the way of selecting, cooking and eating(87). These behavioral changes related to lifestyle may also have a therapeutic benefit(25). The role of diet in mental health could be mediated by inflammatory and oxidative stress pathways(95,96), the modulation of gut microbiota(150) and brain plasticity .

The neurotrophin brain-derived neurotrophic factor (BDNF) is a peptide implicated in a variety of neural processes, including modulation of synaptic plasticity and memory-related mechanism(151). A lower production of this peptide has been observed in patients with depression(151) and also in pregnant patients with poor sleep quality(152). Interestingly, in a sub-group of the PREDIMED study, significantly higher plasma levels of BDNF in participants in the Mediterranean diet intervention arm supplemented with nuts(153). The fatty acid profile of the Mediterranean diet, rich in polyunsaturated fatty acids, may also promote

mental health, as low polyunsaturated fatty acid intake (mainly omega-3 fatty acids) has been associated with depression, among other mental outcomes(154).

So, the synergy between several elements provided by a dietary pattern such as nutrients, bioactive compounds and lifestyle and cultural elements may be required for correct brain function and mental health and that is why dietary interventions may have greater benefits for mental health rather than single nutrients supplementations(155).

Finally, further research to understand the possible mechanism of the impact of the Mediterranean diet and the potential benefits for the pregnancy and offspring outcome is needed.

7.4. Role of the maternal microbiota

Research in last decades on the human microbiome suggests an important role for the microbiota in influencing brain development, behavior and mood in humans(150). Moreover, microbiota has also been postulated as a protective shield in protecting us from exogenous microorganisms through nutrient competition, antimicrobial production and bacteriophage deployment(113).

We wanted to assess the changes in the maternal microbiota as potential mediators of maternal health. To do so, we analyzed the maternal nasopharyngeal microbiota profile in women infected and non-infected by SARS-CoV-2. We found differences in the nasopharyngeal microbial structure and composition: SARS-CoV-2-positive pregnant women showed differences in microbiota richness and evenness, with a higher relative abundance of Bacteroidetes (mainly due to the higher abundance of the *Prevotellaceae* family) and *Tenericutes* phyla. Additionally, we showed that these microbial changes were similar among

women with past and present SARS-CoV-2 infection. No significant differences were reported in the most severe cases.

Ours was the first study to describe the nasopharyngeal microbiota profile in SARS-CoV-2 infection during pregnancy, although studies in non-pregnant COVID-19 individuals found a similar microbial composition in the nasopharyngeal tract to our findings (Firmicutes, Bacteroides, Proteobacteria and Actinobacteria as the most relevant phyla)(156,157). However, previous studies show contradictory results: in a subsample of 18 COVID-19 patients, Nardelli *et a*l. reported differences in beta diversity (reduction in Proteobacteria and Fusobacteria phyla)(158). Rueca *et al.* described a significant reduction in alpha diversity in 19 COVID-19 patients in ICU, without changes due to SARS-CoV-2 positivity(159). In contrast, however, Ventero *et al*. did not find any differences in the richness index between positive and negative cases(156). Authors did report a loss of network complexity and a higher relative abundance of *Prevotella* genus(156). De Maio *et al*., found no differences in bacterial richness, diversity and composition between positive and negative cases(157). A possible explanation for such contradictory findings among the literature could be the sample size, but also different populations and severity of the respiratory symptoms.

As stated above, maternal microbiota has been proposed as a factor to drive fetal development and future infant susceptibility to disease(133). However, we don't have information about how pregnancy could affect nasopharyngeal microbiota in general pregnant population. Our study is a first baseline data about the pregnant women nasopharyngeal microbiota that could be used in future research. However, the lack of this data on the upper respiratory tract microbiota during pregnancy has also been a limitation in our results, as we could not discern if the described changes were due only to the pregnancy status itself and if this could be considered a protective effect for viral infections to become

more severe. Finally, future studies are warranted to compare these data with women of the same age but in a non-pregnant status.

In the same line, we wanted to assess the effects that specific lifestyle interventions during pregnancy could have on the composition and diversity of maternal gut and vaginal microbiota. To do so, we again present the results in the context of a randomized clinical trial involving pregnant women at high risk for an SGA newborn (The IMPACT BCN Trial)(101).

When lifestyle interventions based on Mindfulness stress-reduction techniques and Mediterranean diet were applied during pregnancy, an increased microbial richness in the gut microbiota was found. Moreover, the Stress reduction strategy also impacted in microbial diversity. However, we found no differences in maternal vaginal microbiota.

Existing evidence has shown that a Mediterranean diet could induce changes in the composition, diversity and activity of the gut microbiota(160). In our study, participants in the Mediterranean diet group showed a microbiota enriched in members of *Bacteroides*, *Blautia* and *Bifidobacterium* genera. Members of the genus Bacteroides correspond to a big fraction of the gut microbiome playing multiple roles: protection from pathogens and nutrients supplying to other gut-microbial specie(161). Suggestive associations between an increase in *Blautia* and a lower risk of Alzheimer disease have been reported(162) as well as an inverse correlation with obesity and type 2 diabetes mellitus(163). Finally, *Bifidobacteria* are considered to be among the first microbes to colonize the intestines of new-borns, playing an important role in their development, especially in the maturation of their immune system(164).

Dietary counselling intervention also impacted the alpha-diversity of the pregnant women's microbiota, increasing both the diversity and richness of the gut bacterial community

compared to the non-intervention group. These results are in line with previous studies on the general population following a Mediterranean diet and in some studies on the pregnant population, where the increased consumption of fruits, vegetables and legumes and the low consumption of red meat were key to the association(165).

During pregnancy, studies in animal models have reported differences in the fecal microbial composition of pregnant rodent females when exposed to stress, resulting an increased IL-1β *in utero*, a reduction in BDNF, and long-term alterations in both behavior and microbiome their offspring(166). Bailey *et al.* in their multiple studies concluded that stress has the potential to alter the microbiota leading to bacterial translocation(167).

In our cohort, we observed a significant effect of the Stress-reduction intervention on maternal gut microbiota composition: a significant higher alpha-diversity in this group of participants was observed compared to usual care group. In previous studies, a higher perceived stress was correlated with lower alpha diversity(168). Moreover, a reduced alpha diversity has also been found in infants of mothers who reported high levels of stress and anxiety during pregnancy(169).

We also found a higher relative abundance of *Faecalibacterium* and *Subdoligranulum* genera along with *Lachnospiraceae* and *Ruminococcaceae* families. These results are in line with previous studies assessing the effect of a Mindfulness-based stress reduction program in non-pregnant subjects, which had already described a decreased bacterial diversity among high trait-anxiety populations when comparing to healthy controls. Moreover, a decrease in genera such as *Faecalibacterium* was also described by the authors(170). In contrast, in the same study decreases in *Subdoligranulum* genera post-intervention were indicative of ameliorated anxiety. In previous evidence, an association between low *Lachnospiraceae* and low *Ruminococcaceae* families has been associated to psychological distress(168).

Ours is the first study to show changes in the microbial diversity when applying a structured stress-reduction intervention during pregnancy. These findings could lead to the use of gut biomarkers as outcomes in psychological intervention trials, which could provide a tool to guide treatment or predict response to therapies helping in the individualization and personalization of psychological treatments(171).

When studying the vaginal microbiota in our cohort, we found a low-diversity environment dominated by *Lactobacillus* spp, as expected from previous literature(113). No effect of the interventions on vaginal microbiota was observed in our study.

7.5. Strengths and limitations

This work has several strengths which include the use of different validated questionnaires with clinical applicability to assess mental stress, anxiety, well-being, and sleep quality; which gave us the opportunity to provide an integrative approach of maternal mental health and together with the use of biomarkers in some of our studies may mitigate the potential misclassification of self-reported data, along with the inherent risk of inaccuracies in the measurements, which was a limitation found in other previous published studies. Moreover, these questionnaires were assessed in the second and third trimester of pregnancy, which allowed an analysis of the experimented changes throughout pregnancy in our first study. Also, during the COVID-19 pandemics, we used the short and simple but yet validated WHO-5 questionnaire, that can screen depressive symptoms and evaluate subjective well-being in pregnant population, which can be helpful in daily clinical practice, especially when

healthcare pressure is high as it was during the first wave of the COVID-19 pandemic when the study was conducted.

In our work, we present a very well characterized population of pregnant women, with laboratory confirmation of SARS-CoV-2 infection in all women in different pregnancy stages and during the first wave of COVID-19 pandemic, where strict restriction measures were applied.

Another major strength of our work a very well-characterized population of pregnant women who followed a structured intervention in a randomized clinical trial. In fact, our last study represents the first description from a randomized trial demonstrating an effect of lifestyle interventions during pregnancy on gut microbiota.

Ours was also the first study of nasopharyngeal microbiota in pregnant women, providing data about this specific population and opening the door to future studies focused on them. Moreover, nasopharyngeal Reverse-Transcription Polymerase Chain Reaction (RT–PCR) swab collection was always performed using a standardized procedure from trained medical staff at hospital admission, reducing potential bias before any treatment was started.

Nasopharyngeal samples are considered low-biomass samples, and specific positive and negative controls need to be introduced during sequencing to rule out potential contamination and bias due to the low microbial DNA samples. In this study, specific Amplicon Sequence Variant (ASV) were identified in the negative controls. After filtering process, we further identified specific anaerobic gut microbes, such *Ruminococcus* and *Faecalibacterium*, in the nasopharynx samples, although in lower proportions as other studies also reported to be present in the nasopharynx. Furthermore, the butyrate production of those microbial genera would be associated with a reduction in olfactory function, which has been described as a COVID-19 symptom. Another potential explanation would be that the microbial database

used as the curation of the open databases is critical for proper identification and reliable taxonomy assignment

In contrast, we must also be aware of several limitations: our population was very well characterized but was a high socioeconomic cohort with low ethnical variety and with a low proportion of obesity and gestational diabetes; hence, the results should not be extrapolated to other populations with different characteristics, which makes difficult, on the one hand, the generalization of our results to the general pregnant population and, on the other hand, could also explain some of the findings, especially in sleep disturbances where we could not demonstrate the contribution of these factors in multivariate analysis. Another limitation is that we have no data regarding the first trimester of pregnancy in some of our studies, nor the influence that these negative affective states had on perinatal results. Moreover, neurocognitive function was not assessed, despite its potential influence on mental health(172). In interpreting the results, it is important to understand that the use of self-report instruments may potentially overestimate prevalence, but it is also important to state that they also have high clinical applicability in public health and daily obstetric-care practice. Moreover, baseline characteristics of the pre-pandemic and pandemic cohorts were not identical, although we applied careful statistical adjustments to overcome these limitations.

We must be aware that the intervention trial which provided our results was not designed for the purpose of our study, although maternal stress, well-being and sleep quality were prespecified in the study protocol and assessed from the beginning of the study. We also acknowledge that microbiota was neither the primary outcome of the trial. We were not able to assess long-term dietary intake, including measuring diet before pregnancy or the dietary changes from the beginning of the pregnancy. Our findings should be considered preliminary and require replication, including research involving other study populations and an evaluation of the underlying mechanisms of action.

The relatively small sample size of the microbiota studies, prevented a more comprehensive analysis on the association of microbiota and specific perinatal outcomes in each study group and did not allow us to draw robust conclusions from subgroup comparisons; additionally, as there were no data on the upper respiratory tract microbiota during pregnancy, it was not possible to discern if changes were due only to the pregnancy status itself and if this could be considered a protective effect for viral infections to become more severe.

In addition, the lack of microbiota samples collected before starting the intervention limited the analysis of longitudinal microbiota changes in each participant. Analyses were adjusted by maternal BMI; however, we acknowledge that other potential confounders might have influenced the results. Finally, future studies are warranted to determine the influence of this microbiota changes on perinatal and postnatal outcomes.

8. Conclusions

1. Maternal stress and anxiety, compromised maternal well-being and sleep quality disturbances are frequent and not static throughout pregnancy. Screening for these conditions in different stages of pregnancy should be recommended to professionals providing obstetric care. However, universal screening of this conditions could be challenging: knowing the risk factors can help clinicians target pregnant women at potential risk.

2. Under a negative maternal environment such as the COVID-19 pandemic, maternal mental well-being, especially in their third trimester of pregnancy, is compromised. SARS-CoV-2 infection did not affect the well-being of pregnant women testing positive, but other factors like a previous psychiatric disorder, being in the third trimester of pregnancy or requiring hospitalization due to COVID-19 disease, did.

3. A Mediterranean diet intervention during gestation significantly reduces maternal anxiety and stress, as well as improves well-being and sleep quality. Considering the increasing importance of the role of mental health during pregnancy, these findings might imply the promotion of a pregnancy-adapted Mediterranean diet among pregnant women as a powerful public health strategy to improve mental health during pregnancy.

4. The overall composition and diversity of the nasopharyngeal microbiota differed in pregnant women with and without SARS-CoV-2 infection. These changes were also present in women with a past infection. Further studies are needed to confirm our results and to evaluate the possible clinical implications of nasopharyngeal microbiota

alterations in pregnancies complicated with SARS-CoV-2 infection.

5. The Mediterranean diet and a stress reduction program based on Mindfulness techniques have an effect on maternal gut microbiota, with an increasing in richness and diversity and higher abundance of healthy-associated genera. The maternal gut microbiota could be considered as a potential therapeutic target for compromised maternal mental health.

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