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RESEARCH ARTICLE

Autism Spectrum and gastrointestinal health: Screening on the influence of environmental factors on gastrointestinal problems

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Abstract

Autism spectrum disorder (ASD) is a complex neurodevelopmental condition that combines genetic and environmental factors. The human microbiota is colonized by permanent or transitory microorganisms, depending on the host and the external factors controlling their permanence. The composition of the gut microbiota (GM) in ASD individuals is notably different from that in controls, which may contribute to the clinical conditions observed in these individuals. This study aimed to indirectly investigate the influence of GM on the gut-brain axis in individuals with ASD and controls by analyzing environmental factors that contribute to the microbiota composition. Two questionnaires were designed to collect data, one for the ASD Group (ASDG) and the other one for the Control Group (CG). The raw data from both questionnaires were collected from 2772 respondents. After triage, answers from 1687 ASD individuals, along with 466 respondents from the CG, were analyzed, resulting in a total of 2237 respondents. Our results showed that gastrointestinal problems (GP) escalate as individuals age and become more prominent in ASD individuals. In contrast, feeding problems (FP) did not appear to escalate in either group as individuals aged, even though the FP decreased in the CG. ANOVA revealed significant differences in breastfeeding status compared to GPs among preterm control individuals born via cesarean section (p-value = 0.027). The mean values of GP for breastfed and non-breastfed individuals, for ASDG (0.257; 0.268) and CG (0.105; 0.248), highlighted the differences in breastfeeding effects on GP for the study groups. The use of antibiotics during pregnancy seemed to be significant for GPs in the ASDG only for breastfed individuals (p-value <0.001), but not in the CG group. In conclusion, variables such as mode of delivery, FPs, type of birth, and length of breastfeeding do not seem to be determining factors for GP in the ASDG but are relevant for the CG. However, for ASDG individuals whose mothers took antibiotics during pregnancy, breastfeeding may act as a protective factor, as maternal antibiotic administration during pregnancy seems to aggravate GP-values across the ages of the participants. Considering GP as a proxy for GM and recognizing the importance of GM composition for central nervous system (CNS) function, it

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appears that in individuals with ASD, GM seems to be more dependent on other factors, which might be linked to the genetic background of each one. These findings suggest that future studies of the gut-brain axis in individuals with ASD might consider the individual's genetic background, environmental factors, and GM.

Lay Summary

Autism spectrum disorder (ASD) is influenced by both genetic and environmental factors. This study found that gastrointestinal problems (GP) tend to increase with age in children with autism. Breastfeeding had a protective effect on GP in autistic children whose mothers took antibiotics during pregnancy, while delivery type and breastfeeding duration did not significantly affect GP in autistic individuals. These findings highlight the complex relationship between gut health, environmental factors, and autism, suggesting future research should explore how these interactions impact brain function and behavior.

KEYWORDS

ASD, autism, breastfeeding, gastrointestinal, genetic, gut-brain axis, microbiota, neurodevelopment

INTRODUCTION

Autism spectrum disorder (ASD) is a multifactorial lifelong neurodevelopmental condition (American Psychiatric Association, 2022) that affects social communication and repetitive and stereotyped behavior. It is acknowledged that a genetic predisposition along with environmental circumstances is associated with ASD outcomes (American Psychiatric Association, 2022; Brito et al., 2023; de la Torre-Ubieta et al., 2016; De Rubeis et al., 2014; Hoang et al., 2018; Ng et al., 2017; Vorstman et al., 2017). Although psychiatric and neurological familial history contribute to ASD outcomes, along with intrauterine environmental stress exposure, identifying the mechanisms involved in such contributions is still difficult (Brito et al., 2023).

The gastrointestinal tract and the central nervous system (CNS) are linked through a bidirectional communication network axis, which acts on neural, hormonal, and immune signaling pathways. The gut-brain axis helps modulate neurotransmitter synthesis, immune response regulation, and metabolic homeostasis in the CNS. Dysregulation of this axis has been proposed to be a contributing factor to the core symptoms of ASD, as disruptions in gut-brain communication may lead to altered neural circuitry and neurobehavioral abnormalities (Morton et al., 2023; Pasinetti et al., 2023; Rutsch et al., 2020; Xu et al., 2019). Under stress, the brain triggers various systems that release neurotransmitters, inflammatory factors, and metabolites, influencing gut function. The same mechanism happens when the gut experiences stress, influencing brain function. The HPA (hypothalamic–pituitary–adrenal) axis in the neuroendocrine system also plays a critical role in stress responses by releasing hormones like cortisol. The immune system and gut microbiota (GM) contribute to this communication by producing cytokines and other immune signals that affect both the gut and the brain (Ullah et al., 2023; Wang et al., 2024). Neurodevelopmental disorders, such as anxiety disorder and depression, for example, are associated with more aggressive inflammatory bowel disease, such as Crohn's disease and ulcerative colitis, and symptoms like anxiety and depression are associated with increased disease severity and higher rates of hospitalization (Gao et al., 2021). Besides, anxiety, depression, ASD, and multiple sclerosis might also be possibly influenced by microbial composition on the human brain development, as gut bacteria may impact neurodevelopment through the production of neurotransmitters and modulation of the immune system (Eugenicos & Ferreira, 2021).

The interest in studying the gut-brain axis and the microbiota composition in autistic people has increased because these individuals often present gastrointestinal problems (GP), and there seems to be a link between some genera of bacteria and autism (de Theije et al., 2011; Morton et al., 2023; Pasinetti et al., 2023; Rutsch et al., 2020; Xu et al., 2019). The GM has been frequently noted to play a role in ASD, and some comorbidities and symptoms, such as gastrointestinal disturbances, immune dysfunctions, and food selectivity, are commonly reported in ASD individuals (De Rubeis et al., 2014; Lord et al., 2020). The severity of gastrointestinal symptoms and severe cases of autism were linked once the gut-brain axis showed that altered metabolism could affect neurodevelopment (Fowlie et al., 2018). However, there is still a lack of robust studies investigating this point or showing how brain-gut communication works and may interfere with neurodevelopment (Andreo-Martínez et al., 2022; Cryan & Dinan, 2012; Feng et al., 2023).

Additionally, variables such as mode of delivery (if vaginal or cesarean) and length of breastfeeding are related to microbiota colonization very early in life (Wilson et al., 2023). Vaginal delivery is an essential step

for newborn microbiota colonization but is lacking in cesarean delivery (Korpela et al., 2020). Breastfeeding seems to colonize the human GM through the presence of proteins, oligosaccharides, immune cells, and other components that can transmit beneficial gut bacteria, supporting the metabolic, immune, and neurological systems (Carr et al., 2021).

To better explore the gut-brain axis in individuals with ASD, breastfeeding and GP were used as proxies of GM alterations. Here, we used the data collected from 1687 ASD Brazilian individuals included in our previous study (Brito et al., 2023) and 466 Brazilian individuals with neurotypical development, considered here as the "Control Group (CG)." Both groups answered an online questionnaire containing information related to ASD outcomes, considering maternal and newborn history (Brito et al., 2023). This work aimed to indirectly verify the possible role of the microbiota in ASD individuals by analyzing factors known to contribute to the microbiota composition, such as cesarean and vaginal labor, term and preterm labor, length of breastfeeding, maternal administration of antibiotics during pregnancy, feeding problems (FP), and GP, as proxies for gastrointestinal function in individuals diagnosed with ASD compared to neurotypical individuals. In the present study, the microbiota of the individuals was not analyzed. Instead of collecting stool samples from individuals and analyzing the microbiome, we explored the data related to the type of labor, feeding habits, gastrointestinal issues, and antibiotic administration during pregnancy.

METHODS

Ethics, patient consent, and data collection procedures

All ethical data collection procedures have been approved and described (CAAE 61,093,416.0.00005467) (Brito et al., 2023). In summary, parents of ASD and control individuals, or their legal guardians, signed the consent form after the participants were informed about the project. The data analyzed here were collected through a questionnaire from October 2018 to December 2021. Respondents were invited to answer the questionnaire via the social media of "The Tooth Fairy Project," while attending conferences about autism throughout Brazil. Families with one or more autistic individuals, males, or females, and with a previous ASD diagnosis were accepted. In addition to declaring the ASD, the respondents were also asked to provide a medical report.

Questionnaire and method of analyses

The questionnaire was designed to consider the possibility of ASD presence according to the scientific literature, and it was specially designed for our population in Brazil (Brito et al., 2023). In this work, we explored questions related to FPs, GPs, length of breastfeeding, term or preterm birth, mode of delivery (vaginal or cesarean), and antibiotic administration during pregnancy to indirectly investigate the role of microbiota in the gut-brain axis in individuals with ASD compared with those in the CG.

The "GPs" score was represented by values from one to eight based on the answers for GPs: "Intestinal colic," "Gastric Reflux," "Difficulty Swallowing," "Stomachache," "Other GPs," "Constipation," "Flatulence," and "Frequent Diarrhea." The "FPs" score was represented by values from one to eight based on the answers for FPs: "Seems not to be hungry," "Restrictive eating behavior," "Eats mosetly sweets," "Unhealthy eating habits," "Compulsive eating behavior," "Selective or picky eating behavior," "Problems with texture, color, and smell," and "Healthy eating habits." Every question in the score was weighted as 1.0, except for the "Healthy eating habits" question in the FP score, which was weighted as −1.0, as it represents a positive outcome inside a group of problems.

The survey questions about breastfeeding were divided into four groups: "Breastfeeding from 0 to 6 months", "Breastfeeding from 6 to 12 months", "Breastfeeding for more than 12 months", and "Not breastfed". The questions regarding antibiotic administration included the following options: "Did not take," "Antibiotic administration during the first trimester of pregnancy," "Antibiotic administration during the second trimester of pregnancy," "Antibiotic administration during the third trimester of pregnancy," and "Antibiotic administration throughout the entire pregnancy." For analysis involving antibiotic use, the variable length of breastfeeding was categorized into two groups, "Breastfed" and "Nonbreastfed."

Analyses were performed in Python, using the Pandas library for data management. Levene's test was used to check for equality of variances between GPs in the groups studied. Weighted Logistic Regression, linear regression, and Welch's ANOVA were used to identify significant relationships among the chosen variables, performed using Scikit-learn, Statsmodel for Python, and SciPy libraries (Pedregosa et al., 2011; Seabold & Perktold, 2010; Virtanen et al., 2020).

RESULTS

Characterization of the participants

The raw data from the questionnaire comprised 2237 respondents from the ASD Group and 490 from the CG. Participants who could not answer any of the questions required for this study were excluded from the analysis, resulting in a final dataset with 1687 respondents from the ASD group and 466 from the CG. To

TABLE 1 Sample characterization. Questions about ASD level, sex, age, term or preterm delivery, and mode of delivery were used to construct the final dataset.

		ASD	Control
ASD level	Level 01	888	-
	Level 02	662	-
	Level 03	137	-
Sex	Male	1381	258
	Female	306	208
Age	Mean	7.8y	9.6y
Term or Preterm delivery	Term	1075	311
	Preterm	612	155
Mode of delivery	Cesarean	1344	352
	Vaginal	307	105
	Other	36	9

Note: Mode of delivery: "Other" refers to deliveries such as "Cesarean with forceps," "Vaginal with forceps," or any method that differs from the most common forms of regular vaginal or cesarean delivery.
Abbreviation: ASD, autism spectrum disorder.

characterize the respondents, we used answers to questions related to sex, age, term or preterm delivery, and mode of delivery. The respondents' characteristics are described in Table 1. The ASD Group presented all three ASD levels (Levels 01, 02, and 03), with 53% under Level 01 (APA, 2022). ASD levels were used solely as inclusion or exclusion criteria for participants to ensure the reliability of the responses. The final dataset included only answers from respondents who provided their ASD level, excluding those who answered "I do not know." ASD levels were classified by various professionals, as the respondents were from all states in Brazil. Given that this classification could vary, ASD levels were not used as indicators of severity in this study.

Regarding sex in the ASD Group, most participants were males, which was 4.5 times more common than females; these findings are similar to what has been described in the scientific literature (Brito et al., 2023; Maenner et al., 2023). However, this imbalance between males and females was not detected for the CG, which was 1.24 times more common for males than females (Table 1). The average ages of the participants in both groups were similar (ASDG: 7.87 y, 95% CI [7.62; 8.12]; CG: 9.62 y, 95% CI [8.97; 10.26]). Considering birth information, both groups presented more newborns delivered at term than at preterm, and most of them were delivered by cesarean section, a more common mode of delivery in Brazil (Oliveira et al., 2016). Levene's test for equality of variances was significant for GPs when compared between the ASDG and CG, indicating that Welch's ANOVA would be a more appropriate choice for analyzing the variables, as it is suited for hypotheses where equal variances are not assumed, unlike standard ANOVA. Given the class imbalance between ASDG and CG responses, weighted logistic regression was used to test the significance of binary variables related to the presence of ASD.

Analyses of "GPs" and "FPs"

A weighted logistic regression model was applied to identify the significant variables among the multiple answers related to GPs and FPs, highlighting the most critical symptoms associated with ASD and their respective odds ratios (OD) (Figure 1). For GPs related to individuals in ASDG, the binary answers for the presence of "Constipation," "Flatulence", "Difficulty swallowing", "Stomachache," and "Frequent diarrhea" were significant (p-value <0.01). For FPs, only the binary answers for the presence of "Seems not to be hungry" and "Unhealthy eating habits" were not significant (p-value >0.05).

Analyses of "GPs score," "FPs score," and "breastfeeding" status

The dataset, consisting of 1687 valid answers from the ASD Group and 466 from the CG, was divided into subgroups of interest for ANOVA (Figure 2a). This subdivision was created to determine under which circumstances breastfeeding, a proxy for the intestinal microbiota, could influence GPs. The effects of breastfeeding were compared to the GP score within each subgroup (Figure 2b), revealing a significant difference in incidence among preterm individuals born via cesarean section in the CG (p-value = 0.027), which was not observed in the ASD Group in the hypothesis testing.

The GPs and FPs were significantly different than those associated with the presence of "ASD" (p-value <0.001). GPs and FPs were linearly scaled (GPs and FPs = [0,1]). The mean of the scores for every age in the dataset was computed to determine how the scores behave across ages (Figure 3). An increase in GPs was observed as age increased for the ASDG ($\beta = 0.006$, 95% CI [0.004; 0.007]) and CG ($\beta = 0.002$, 95% CI [−0.002; 0.006]), reaching higher values for the ASDG (0.250, 95% CI [0.197; 0.303]) than for the CG (0.101, 95% CI [0.052; 0.150]). For the FPs, ASDG (0.348, 95% CI [0.289; 0.407]) presented a mean value far above that of the CG (0.120, 95% CI [0.083; 0.157]).

For the ASDG, the GPs increased with age (Figure 3a), which was not followed by the FPs (Figure 3c). For the CG, the GPs' means also increased with age (Figure 3b). In contrast, the mean FPs decreased (Figure 3d). As FPs do not seem to evolve as GPs mean, FPs might not be directly associated with the evolution of GPs in both groups as individuals age.

Once statistically significant within the CG, the cesarean section subgroup was selected to inspect the influence of breastfeeding on GPs without any differentiation of term or preterm birth. The GP score was also

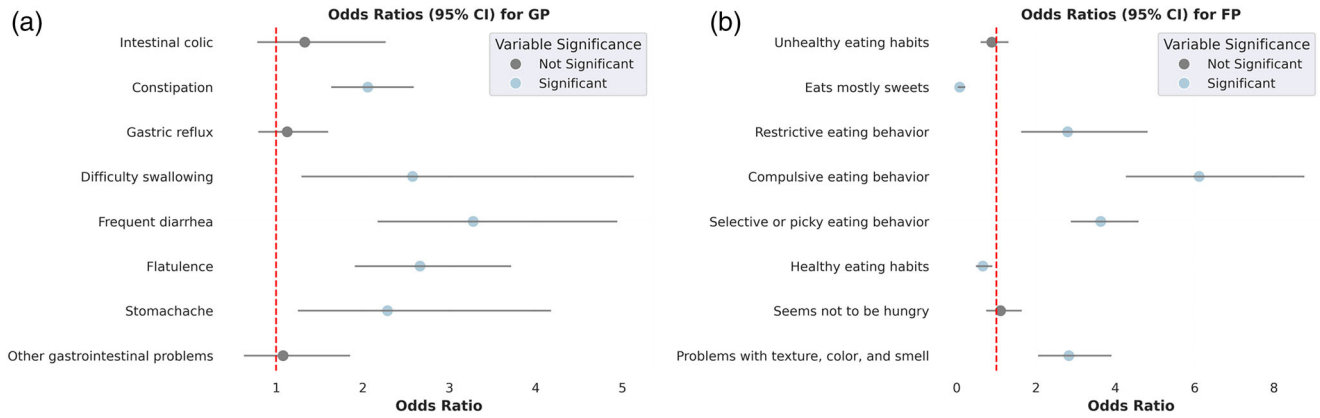

FIGURE 1 Odds ratios (OR) from weighted logistic regression for GP and FP related to the presence of ASD. (a) OR for variables associated with GP, categorized as "significant" or "not significant." (b) OR for variables related to FP, classified as "significant" or "not significant." ASD, autism spectrum disorder; FP, feeding problem; GP, gastrointestinal problem.

analyzed by the length of breastfeeding and cesarean delivery (Figure 4). Despite the increase in regression coefficients from breastfed individuals (ASDG: $\beta = 0.0003$, 95% CI [−0.006; 0.006]; CG: $\beta = 0.001$, 95% CI [−0.005; 0.007]) to nonbreastfed individuals (ASDG: $\beta = 0.006$, 95% CI [0.002; 0.010]; CG: $\beta = 0.011$, 95% CI [0.001; 0.021]), only CG presented a significant relationship between GPs and length of breastfeeding when compared to the same condition. Breastfed individuals in the ASDG (0.257, 95% CI [0.202; 0.312]) and nonbreastfed individuals in the ASDG (0.268, 95% CI [0.201; 0.335]) did not present relevant differences in the GPs means. Moreover, it is possible to identify important differences between breastfed individuals in the CG (0.105, 95% CI [0.042; 0.168]) and nonbreastfed CG individuals (0.258, 95% CI [0.138; 0.378]), suggesting a positive impact of breastfeeding across groups of ages in the CG but not in the ASDG.

For breastfed individuals, the GPs means had slight variation according to ASDG age (Figure 4a) and CG age (Figure 4b). For nonbreastfed individuals, the GPs means age increased concerning the ASDG age (Figure 4c) and CG age (Figure 4d). These results suggest a positive effect of breastfeeding on GP, as the GPs mean from breastfed individuals is lower. Although the ASDG also seems to be impacted positively by breastfeeding, ANOVA results were not significant for the group, and the GPs means remained similar (breastfed individuals: 0.257; nonbreastfed individuals: 0.268), suggesting that breastfeeding would not interfere with the GP for ASD individuals.

The GP score was also analyzed regarding antibiotic administration during pregnancy. A linear regression model was applied to identify the significant variables among the multiple answers related to the use of antibiotics during pregnancy (Table 2), which highlighted the significant relationship between GPs and antibiotic administration during the first and second trimesters of pregnancy (p-value <0.001). The overall model was statistically significant ($F(4, 1557) = 8.921$, $p < 0.001$), explaining approximately 2.2% of the variance in GPs ($R^2 = 0.022$). The relatively low R-squared value indicates that other factors contribute to GP, and future research should explore additional predictors. In addition to the regression test, an ANOVA test was used to identify the influence of antibiotics on GPs within ASDG (p-value <0.001) and CG (p-value = 0.774), showing significant results only for the ASDG. Breastfed and nonbreastfed condition, in co-occurrence with the administration or not of antibiotics, was also verified (Breastfed: p-value <0.01; Nonbreastfed: p-value = 0.255), presenting statistical significance only for the individuals from ASDG who were breastfed, indicating that antibiotics during pregnancy could affect the outcome of GPs in autistic individuals and that breastfeeding could also interfere in this relationship.

To analyze the possible relationship of GPs with antibiotics and breastfeeding within the ASDG, regression coefficients were obtained from a linear regression involving GPs (Figure 5). It is notable that, in this context, breastfeeding emerges as a potential protective factor as its presence gradually reduces the coefficient β, from Figure 5a–c, on the different combined conditions. For the most unfavored scenario, where there was the use of antibiotics and no breastfeeding ($\beta = 0.023$, 95% CI [0.009; 0.038]), passing through an intermediate scenario, where there was the use of antibiotics and the person was breastfed ($\beta = 0.014$, 95% CI [0.003; 0.025]), until the most favorable scenario, where there was no antibiotics administration during pregnancy and the person was breastfed ($\beta = 0.004$, 95% CI [−0.002; 0.010]).

DISCUSSION

The human microbiome consists of a complex population of microorganisms, and their DNA inhabits the central mucosal tissue and skin in the human body, impacting

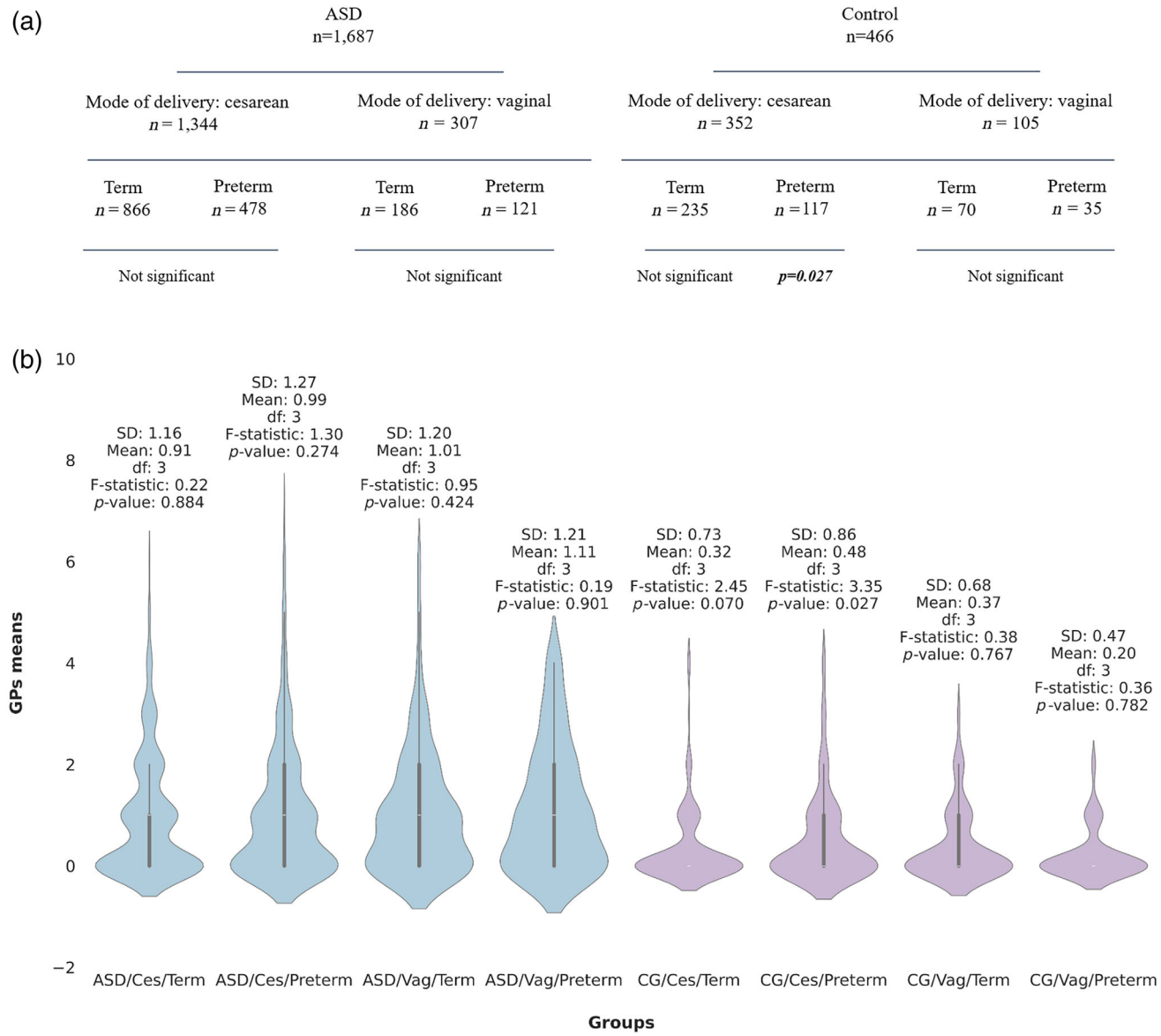

FIGURE 2 Effect of breastfeeding on GPs given subgroups of individuals according to their ASDG and CG, term or preterm birth, or mode of delivery. (a) Subgroup divisions for ANOVA. The ASDG ($n = 1687$) and CG ($n = 466$) were divided by mode of delivery, followed by term or preterm birth. The GP score was set as a dependent variable for hypothesis testing of the effects of breastfeeding, presenting a significant influence of breastfeeding on GPs of preterm and cesarean-born individuals from CG (p-value = 0.027). (b) Violin plot showing the distribution of GPs by a subgroup of analysis. ASDG, autism spectrum disorder Group; CG, Control Group; GP, gastrointestinal problem.

human homeostasis (van der Meulen et al., 2016). The bidirectional gut-brain axis may be part of a series of other physiological systems (immune, neurological, and metabolic) influenced by one's microbiota function. Microorganisms can interact with or cross the gut epithelial barrier, which may disrupt an individual's homeostasis, depending on the composition of the GM. In addition to the gut, studies of the womb, cutaneous, vaginal, lung, and oral microbiomes are underway (Mathieu et al., 2018; Pasinetti et al., 2023; Taddei et al., 2018). This bacterial colonization in tissues and organs is based on a symbiotic relationship, and the microorganism may be permanent or transitory, depending on the host and the external factors controlling its permanence (Pasinetti et al., 2023; Sekirov et al., 2010).

It is known that there might be a relationship between the human microbiome and psychiatric/neurological conditions, such as depression, anxiety, attention deficit hyperactivity disorder, and ASD (Galland, 2014; Felice and O'Mahony, 2017; Osadchiy et al., 2019). In addition, the regulation and maintenance of intestinal barrier function protects against infections and promotes the tolerance of foods in a process known as eubiosis (Sekirov et al., 2010). New findings about the microbiome-gut-brain axis describe how metabolites from the microbiota can reach the brain, altering the

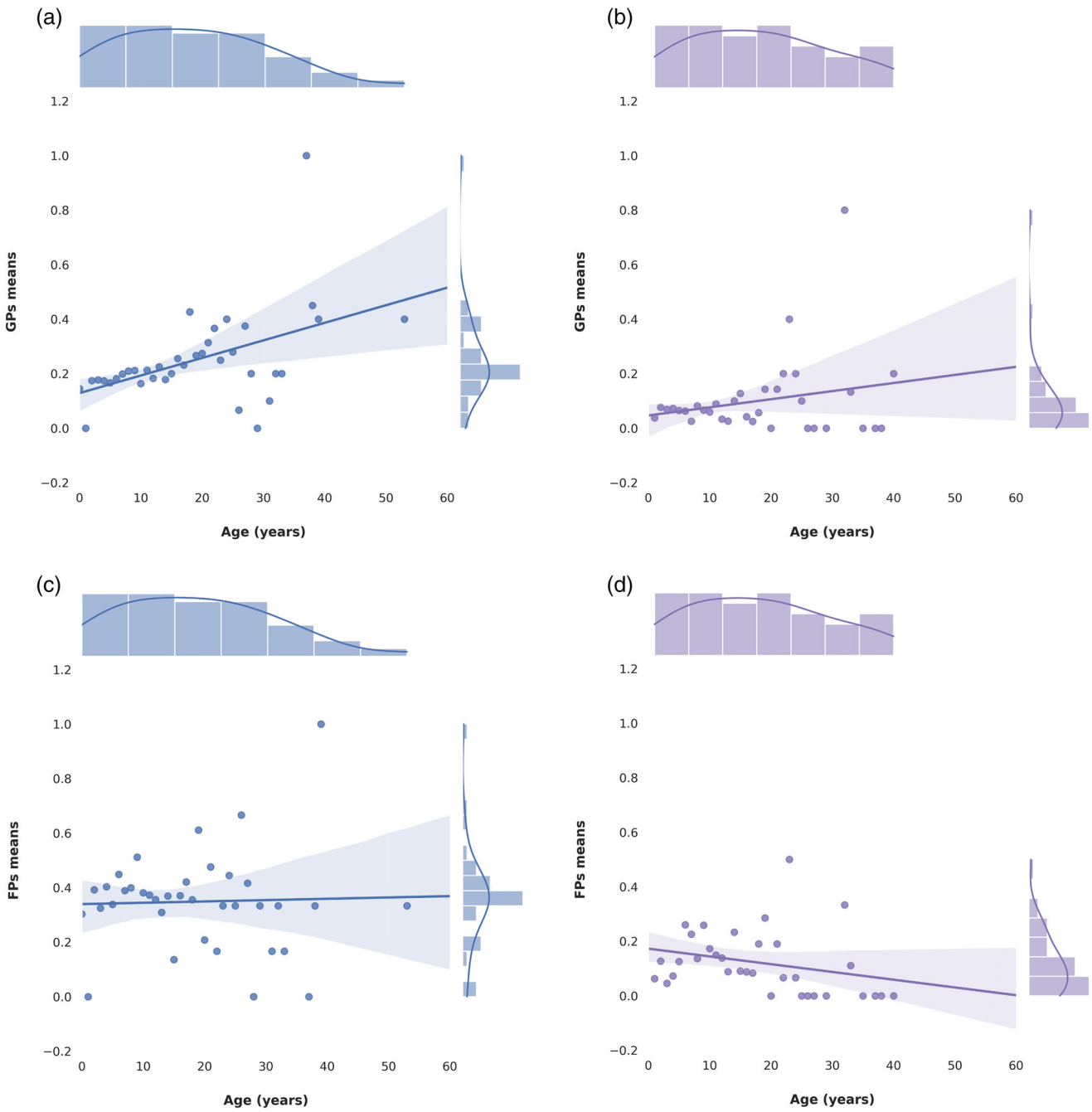

FIGURE 3 Linear regression of ASDG and CG considering GPs and FPs means by age. (a) Linear regression of ASDG GPs means over the variable "Age." (b) Linear regression of CG GPs means over the variable "Age." (c) Linear regression of ASD Group FPs means according to age. (d) Linear regression of the CG FPs means versus the variable "age." The histograms in the figures are placed for GPs means, FPs means, and "age" to illustrate the range of most frequent values across the variables. Across ages, the GPs means had a positive slope for both groups, with a slightly greater slope, and it was assumed that mean values were more elevated for ASDG than for CG. In contrast, the FPs presented low variation across ages for ASDG and a negative slope for CG, indicating that the FPs had lower means across the ages of the participants. ASD, autism spectrum disorder; ASDG, autism spectrum disorder Group; CG, Control Group; GP, gastrointestinal problem.

production and function of neurotransmitters both in eubiosis and dysbiosis situations (Fasano, 2020; Vuong et al., 2017). In addition, the induction of the immune system by intestinal microbiota members can increase the production of inflammatory cytokines with neurological effects (Li et al., 2017; Osadchiy et al., 2019).

Studies on the GM and ASDs have suggested that there might be a correlation between these two conditions. However, robust data are needed to confirm this correlation and to better understand how the gut-brain axis functions. Our data indicate that ASD individuals have more GPs than individuals in

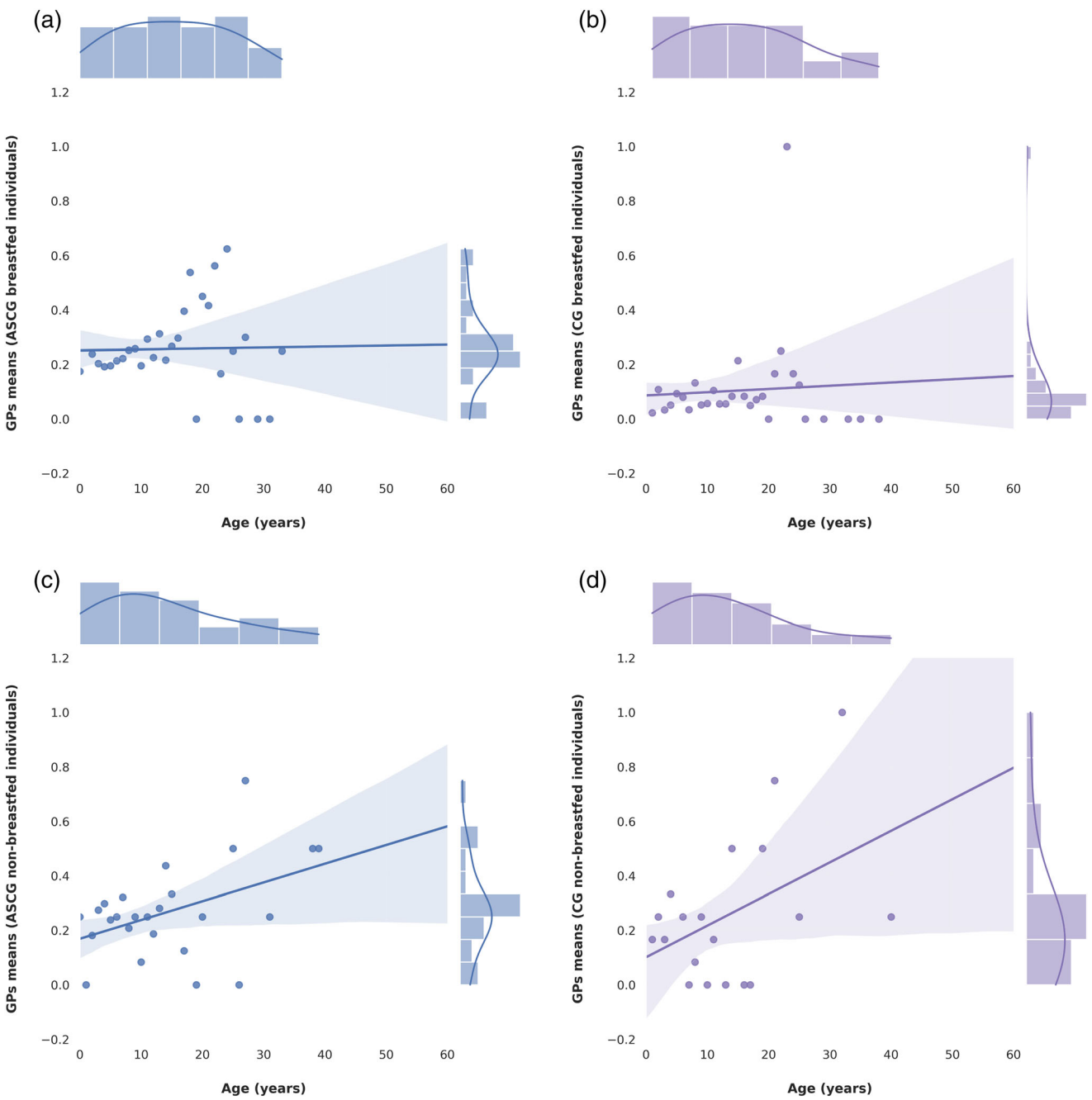

FIGURE 4 Linear regression of ASDG and CG scores considering the gastrointestinal problem score means (GPs) by age within the subgroup mode of delivery (cesarean) was performed to evaluate the effects of breastfeeding on GP incidence. (a) Linear regression of ASDG GPs means over the variable "Age," considering only breastfed individuals born from cesarean delivery. (b) Linear regression of CG GPs means over the variable "Age," considering only breastfed individuals born from cesarean delivery. (c) Linear regression of ASDG GPs means over the variable "age" considering nonbreastfed individuals born from cesarean delivery. (d) Linear regression of CG GP means over the variable "Age," considering nonbreastfed individuals born from cesarean delivery. The histograms illustrate the range of most frequent values across the variables. The regression coefficient changes for a higher value for both groups, ASDG and CG, both breastfed and nonbreastfed. Nevertheless, the relationship between GPs and breastfeeding was significant only within the CG. ASD, autism spectrum disorder; ASDG, autism spectrum disorder Group; CG, Control Group; GP, gastrointestinal problem.

the CG do, and these problems worsen as they age, independent of the mode of delivery or breastfeeding conditions. In addition, the analysis of the CG revealed that GPs were correlated with the mode of delivery and breastfeeding. In the CG, GPs were increased in preterm children born by cesarean delivery and in those who were not breastfed. Our findings corroborate previous studies in which breastfeeding was negatively correlated with GPs (van den Elsen et al., 2019).

The complementary analysis conducted to investigate the potential effects of maternal antibiotic administration during pregnancy on GP for individuals in ASDG and CG revealed that the use or non-use of antibiotics significantly influenced the GP of individuals in ASDG but not in CG. When breastfeeding was included in the evaluation, the relationship between GPs and antibiotic use remained significant only for the breastfed ASDG, indicating that breastfeeding may have a protective effect for individuals with ASD when maternal antibiotic use occurs during pregnancy. It is known that maternal antibiotic use is associated with a higher risk of autism (Njotto et al., 2023), however, the extent of these interactions in GM has yet to be determined. The findings also suggest that antibiotic use during the 1st and 2nd trimesters is associated with higher GPs in offspring.

TABLE 2 Results from the hypothesis testing on the influence of antibiotic use during pregnancy upon the gastrointestinal problems score. The responses regarding antibiotic use during pregnancy were subjected to hypothesis testing to identify any significant relationship with GPs within the ASDG and CG and the presence of ASD, separately.

Antibiotics questions	GPs for ASDG (p-value)	GPs for CG (p-value)
• Antibiotic administration throughout the entire pregnancy	0.082	> 0.05
• Antibiotic administration during the first trimester	< 0.001	> 0.05
• Antibiotic administration during the second trimester	< 0.001	> 0.05
• Antibiotic administration during the third trimester	0.215	> 0.05

Abbreviations: ASDG, ASD Group; CG, Control Group; GPs, gastrointestinal problems.

The alteration of the GM composition in individuals with intestinal disorders such as flatulence (Manichanh et al., 2014) and diarrhea (Li et al., 2021) was previously described. However, the exact mechanism of these disorders has yet to be fully elucidated. Nevertheless, it seems that the GM and constipation are not fully related (Li et al., 2021; Lai et al., 2023). These previous data and the results presented here allow us to suggest that the modulation of gastrointestinal function in individuals with ASD might be related to other factors besides environmental, such as the genetic composition of each individual. Additionally, our findings contribute to the understanding that external factors such as breastfeeding, nutrition, antibiotic administration during pregnancy, and type of birth might not solely influence the modulation of the GM in individuals with ASD.

LIMITATIONS

Although our group did not diagnose the participants, questions were designed to assess the diagnosis's reliability, as presented in the "Materials and Methods" section. Respondents were also asked to send reports signed by doctors to ensure a diagnosis.

In our previous work (Brito et al., 2023), we considered counting on one's memory to answer environmental

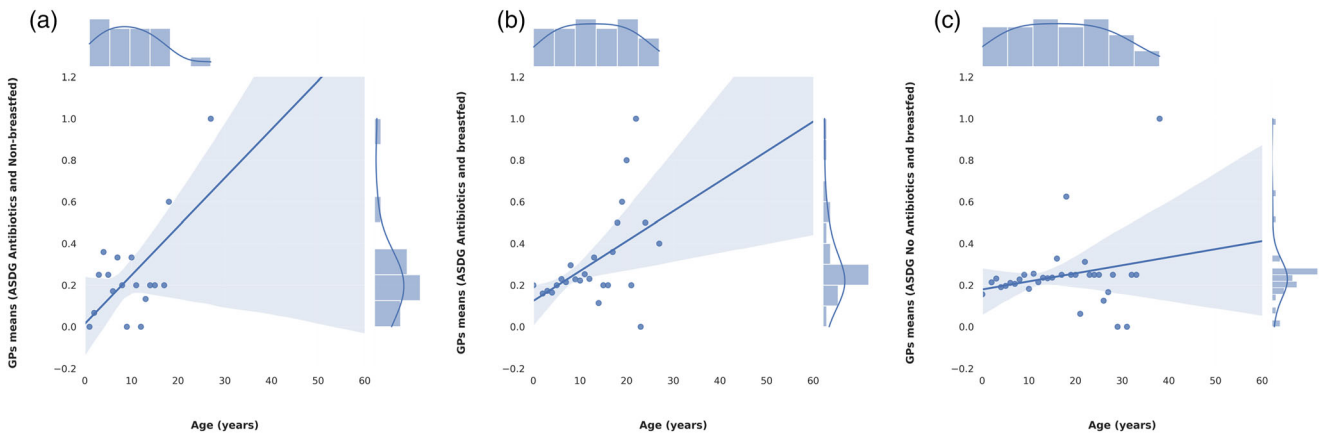

FIGURE 5 Linear regression of ASDG and CG scores considering the gastrointestinal problem score means (GPs) by age, antibiotics use during mother's pregnancy, and breastfeeding on GP incidence. (a) Linear regression of ASDG GPs means over the variable "Age," considering Nonbreastfed individuals whose mothers took antibiotics at least once during pregnancy. (b) Linear regression of CG GPs means over the variable "Age," considering breastfed individuals whose mothers took antibiotics at least once during pregnancy. (c) Linear regression of CG GPs means over the variable "Age," considering breastfed individuals whose mothers did not take antibiotics during pregnancy. The histograms illustrate the range of most frequent values across the variables. The regression coefficient β changes from a higher value (a, b) to a lower value (c) for the ASCG, indicating an intensified effect in the GPs for individuals whose mothers took antibiotics during pregnancy. The breastfed condition seems to attenuate the GPs for individuals whose mothers did not take antibiotics, showing that breastfeeding might influence the GP for ASDG when specific external factors are taken into account, working as a potential protective factor. ASD, autism spectrum disorder; ASDG, autism spectrum disorder Group; CG, Control Group; GP, gastrointestinal problem.

questions as a problem, as one might have a false or blurred memory remembering past events, mainly if they happened more than five (or more) years ago. However, we have not received any complaints about that. In this work, the answers about FPs and GPs may be more recent than gestation events, which may not have yielded false information. To maintain the reliability of our data, answers "I do not know" were excluded from our analysis. Hence, as differences between the included and excluded respondents were not tested, this could be a limitation of our work.

The genetic background was assessed through family history, which is not enough to say that the issues found in our data are due to genetic problems. Another area for improvement is regarding Internet connections, as a few participants reported needing help filling out their forms using a cell phone or in places with poor Internet connections.

FUTURE DIRECTIONS

Considering our results in this work, it is worth mentioning that therapeutic approaches aimed at improving the microbiota may not interfere with the outcome of ASD or the ASD phenotype in our study population. However, they are important for individuals' well-being. Future research is highly encouraged in other populations. Genetic and microbiome tests are being considered for our next steps.

COMMUNITY INVOLVEMENT

This work was possible due to the involvement of parents/caregivers of autistic people and the active participation of adult-autistic people. Community service providers, such as biologists, neuroscientists, psychologists, and psychiatrists, were directly involved in helping to elaborate the questionnaire in a way that was practical and understandable. Notably, all professionals involved in this research were directly involved in treating and researching autism. Hence, their expertise and helpful input reflected their professional experiences rather than their lived experience.

AUTHOR CONTRIBUTIONS

A. B., F. R. T., C. R. T., and P. C. B. B. B. conceptualized the study. A. B. conducted the interviews and collected all the data. F. R. T. and A. F. analyzed the data. A. B., F. R. T., C. R. T., H. B., and P. C. B. B. wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are not publicly available due to the disclosure of respondents' data, such as names and other private information, but they are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Ethical approval for the study was granted by the Ethical Committee of the Institute of Biomedical Sciences from the University of São Paulo, Brazil, #2.945.213/CEPSH. All methods were carried out under relevant guidelines and regulations. Informed consent was obtained from all the subjects and/or their legal guardian(s) when they signed up for informed consent. Importantly, no experiment was carried out on any of the participants; only one online questionnaire was completed.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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