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Title

A meta-analysis of gut microbiota in children with autism

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Abstract

Previous studies have reported dysbiosis in the gut microbiota (GM) of children with autism spectrum disorders (ASD), which may be a determining factor on child development through the microbiota-gut-brain axis. However, it is not clear if there is a specific group of dysbiotic bacteria in ASD. The aim of this study was to carry out a meta-analysis on the studies that analyze GM in children with ASD. 18 studies fulfilled our selection criteria. Our results showed a lower relative abundance of *Streptococcus* (SMD_+ = -0.999; 95%CI: -1.549, -0.449) and *Bifidobacterium* genera (SMD_+ = -0.513; 95%CI: -0.953, -0.073) in children with ASD. Overall, the *Bifidobacterium* genera is involved. However, differences found between studies are attributed to factors such as reporting bias.

Keywords: Autism Spectrum Disorders (ASD); Gut Microbiota; Microbiota-gut-brain axis; Systematic review; Meta-analysis

Introduction

Autistic Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social communication, social interaction, as well as repetitive and restricted patterns (APA 2013).

Various studies have indicated the comorbidity of nutritional problems (Sharp et al. 2013) and gastrointestinal symptoms in ASD (McElhanon et al. 2014). Specifically, there is a higher prevalence of gastrointestinal symptoms (GI), such as diarrhea, constipation, and abdominal pain, in children with ASD compared to other healthy children (HC) (McElhanon et al. 2014). The microbiota-gut-brain axis hypothesis tries to explain the relationship between GI, gut microbiota (GM) (Mayer et al. 2019) and ASD symptoms (Herd et al. 2018). Therefore, the microbiota-gut-brain axis is defined as a bidirectional communication system between the neuronal, immune, endocrine and metabolic pathways. Recent scientific literature has attempted to determine if there is a group of bacteria directly involved in ASD, or if there is general dysbiosis in the GM of children with ASD (Andreo-Martínez et al. 2019; Vuong and Hsiao 2017). However, despite recent studies published on the subject, the etiology of nutritional and gastrointestinal problems in children with ASD is still unknown (McElhanon et al. 2014) and everything seems to indicate that it involves a conjunction of various associated factors (Andreo-Martínez et al. 2019; Martínez-González and Andreo-Martínez 2019a). Specifically, the interactions between environmental (e.g., cultural and dietary aspects, effects of the antibiotics, etc.) and genetic factors (e.g., comorbidity with intellectual disability, etc.) are relevant variables. As a consequence, a defective immune system determined by epigenetic transcriptional factors might be involved in the appearance of ASD (Andreo-Martínez et al. 2019).

Environmental factors and key genes, together with neurological alterations associated with atypical neural growth in children with ASD during the uterine period (Bonnet-Brilhault et al. 2018) can also be associated with GM dysbiosis. GM maturation occurs during the first years of life, together with the critical window of early brain development, which indeed is an important period for the appearance of neurodevelopmental disorders (Diaz Heijtz 2016; Wang et al. 2018). On the other hand, there is an interaction between GM and the epithelial cells of the gastro-intestinal tract. Therefore, GM seems to act as an epigenetic regulator of several diseases (Kumar et al. 2014). Other authors suggest that abnormal GM in ASD may be due to the overuse of antibiotics (Krajmalnik-Brown et al. 2015).

Epigenetic transcriptional factors, metabolites (propionic acid or PPA; short chain fatty acids or SCFAs), lipid and mitochondrial metabolism, and gaseous molecules, ion channel/gap junction/transporter regulation protein, and post-translational modification have also been analyzed (Heuer et al. 2019). In addition, other biomarkers, such as the neurotransmitters implicated in the enteric nervous system (dopamine or DA; norepinephrine or NE; epinephrine or E; serotonin 5-HT; GABA), have also been related to etiology and behavior in mental disorders such as ASD (Kang et al. 2018; Ooi et al. 2017; Dall'Aglio et al. 2018).

Review of previous meta-analyses on the relationship between microbiota and ASD

To date, two meta-analyses have been published on the relationship between GM and ASD (Iglesias-Vázquez et al. 2020; Xu et al. 2019). In Iglesias-Vázquez et al.'s (2020) meta-analysis, 18 studies assessing association between GM and ASD were integrated. A significantly larger abundance in the GM of children with ASD was found for the following phyla: *Bacteroidetes, Firmicutes,* and *Proteobacteria*. Further, they found

significantly greater abundance in the following genera: *Faecalibacterium*, *Bacteroides*, *Parabacteroides*, *Clostridium*, and *Phascolarctobacterium*. In addition, they found lower abundance in children with ASD in the *Bifidobacterium* and *Coprococcus* genera. It is important to note that in Iglesias-Vázquez et al.'s (2020) meta-analysis, each primary study was divided into two analysis units (ASD and control groups), obtaining one overall relative abundance for the ASD groups through the studies, and another overall relative abundance for the control groups. Then, by means of subgroup analysis, these two overall relative abundances were compared. The synthesis method applied in Iglesias-Vázquez et al.'s (2020) meta-analysis is discouraged, as each ASD group must be compared to *its own* control group in order to avoid potential confounding effects known as Simpson's paradox (Borenstein et al. 1999; Rücker and Schumacher 2008). To integrate the results of a set of studies, adequate meta-analytic methods imply: (a) to calculate an effect size from each individual study (e.g., a mean difference or a standardized mean difference that compares the average abundance of ASD and control groups), and (b) to statistically integrate these effect sizes with the purpose of obtaining a pooled effect size, to construct a confidence interval, to assess heterogeneity, and to search for moderator variables than explain the heterogeneity (Borenstein et al. 2009; Cooper et al. 2019).

Xu et al. (2019) carried out another meta-analysis of 9 studies that investigated the association between GM and ASD. They found significantly lower abundance in ASD groups of the following genera: *Akkermansia*, *Bacteroides*, *Bifidobacterium*, *E. Coli*, and *Enterococcus*. In addition, they found greater abundance in ASD groups of the *Faecilobacterium*, *Ruminococcus*, and *Lactobacillus* genera, and they applied the same synthesis method that in Iglesias-Vázquez et al. (2020).

Objective

Although two meta-analyses on the association between microbiota and ASD have been published recently, their results are inconsistent. Xu et al. (2019) meta-analysis included 9 published studies until July 2017, such that the degree of overlapping with our meta-analysis is just 38.8%. In addition, the low number of studies prevented them from carrying out moderator analyses. On the other hand, Iglesias-Vázquez et al. (2020) meta-analysis included 18 studies, but not exactly the same studies as us. Although the degree of overlapping with this meta-analysis was 72.2%, our meta-analysis offers several important improvements. First, in our meta-analysis, a more adequate statistical synthesis of the study findings was applied, based on the comparison between ASD and control groups for each primary study, instead of separately aggregating relative abundances for ASD and control groups. Second, we applied moderator analyzes to identify study characteristics associated with the effect sizes. Third, we analyzed the potential influence of publication bias on the results. Fourth, a re-calculation of the effect sizes and synthesis methods of the two previous meta-analyses was accomplished in order to enable their comparison with those obtained in our meta-analysis. Thus, the objective of the present study was to carry out a systematic review and meta-analysis of the bacteria involved in children with ASD, as compared to healthy controls. We were also interested in identifying potential moderator variables of the heterogeneity exhibited by the study results, and trying to explain discrepancies in the results found in previous meta-analyses.

Methods

This systematic review and meta-analysis was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines

(Moher et al. 2009). Supplementary Table S1 presents the PRISMA checklist for this systematic review.

Selection criteria of the studies

The inclusion criteria were: 1) studies conducted on a human population; 2) articles published from inception to 27th January 2020; and 3) studies comparing the GM of children with ASD with control groups. The exclusion criteria were: 1) descriptive reviews, systematic reviews and meta-analysis; 2) dissertations and proceedings of conferences; 3) books or book chapters; 4) editorial material, letters to the editor, thesis, and shorts reports; 5) studies in vitro and animal model studies; 6) other diseases and intervention studies; 7) articles studying only metabolites in blood, plasma or urine, and genes; and 8) articles published in a language other than English.

Search strategy

The comprehensive databases used were: Scopus, Web of Science, Science Database, and PubMed. The Boolean strings chosen were: (gut* OR intestine* OR bowel* OR gastrointestinal*) AND (microbiota* OR microflora* OR bacteria* OR microbiome* OR flora* OR bacterial* OR bacteria* OR microorganism* OR feces* OR stool*) AND (autistic* OR autism* OR ASD*). The searches included works published in all languages. Scopus database options search were: “title, abstract and keywords”. Web of Science database option search was “theme” in all databases. Science Database and PubMed database option search was “all fields”.

Data extraction and assessment of study quality

A protocol for extracting the characteristics of the studies was produced. The following characteristics were extracted: sample size of ASD and HC groups, percentage of women in ASD and HC groups, mean age of autistic and control participants, percentage of autistic and healthy controls with gastrointestinal problems (constipation

problems, diarrhea and abdominal pain), use of probiotics (living non-pathogenic microorganisms), diagnostic procedure for ASD group, severity level of ASD, comorbidity (ASD with or without intellectual disability), DNA extraction area (feces, ileal mucosa and rectum mucosa), DNA extraction method (culture independent method, standard culture-based method and mixed), balance between the sample sizes of ASD and HC groups, reporting bias, continent, and publication year.

The methodological quality of the included studies in the meta-analysis was assessed with 8 items of the *Newcastle-Ottawa Scale* (NOS) for case-control studies (Wells et al. 2015). This scale uses a 'star system' to judge on the basis of three dimensions: selection, comparability and exposure for case-control studies. The NOS consists of 8 items and the total maximum quality score is 10 stars.

To assess the reliability of the extraction of the study characteristics, as well as the quality items of the NOS, all studies were doubly coded by two independent researchers and the inconsistences were resolved by consensus. For categorical variables, kappa coefficients ranged between .770 and 1.0 (M = .929), and for continuous variables all intra-class correlations were equal to 1.0.

Computation of effect sizes

In this meta-analysis, the effect size index used was Hedges' standardized mean difference (*SMD*), defined as the difference between the mean of the ASD group and the mean of the control group, divided by a pooled standard deviation (*S*): $SMD = c(m)(\bar{y}_{ASD} - \bar{y}_C)/S$, with $c(m)$ being a correction factor for small sample sizes (Hedges and Olkin 1985). Positive *SMD* values indicated a larger mean abundance of bacteria detected in the ASD group than in the control group. For standardization, *SMD*s around 0.2, 0.5, and 0.8 were categorized as small, moderate, and large

magnitudes, respectively (Cohen 1988). For the effect size calculations, we used the available means (both relative and absolute abundance) and the standard deviations for each group. In some studies, this information was not reported, and the corresponding authors of these studies were contacted to request the required missing data. If no reply was received, the effect sizes were calculated using conversion equations from significance tests (e.g., *t*-test and U-Mann Whitney test) and sample size (Borenstein et al. 2011). When the results were reported by means of odds ratio, a conversion formula was applied to obtain the corresponding *SMD* value (Sanchez-Meca et al. 2003). When a study applied several methods for extracting bacteria on fecal samples (e.g., on ileal and ceca mucosal biopsy), a *SMD* index was calculated for each method. Then, in order to avoid dependence problems, they were averaged to represent the specific study. Separate effect sizes were calculated for each bacteria detected (phyla and genera).

Statistical analyses

Separate meta-analyses were carried out for the *SMD* of each bacterial phyla and genera in at least 4 studies, by assuming random-effects models. This model involves weighting each effect size by its inverse variance, defined as the sum of within-study and between-studies variances, estimated by restricted maximum likelihood (Cooper et al. 2019). For each bacteria analyzed, a forest plot was constructed and a weighted mean effect size with a 95% confidence interval (CI) was computed with the improved method proposed by Hartung (1999); (Sánchez-Meca and Marín-Martínez 2008). To assess the heterogeneity exhibited among the effect sizes, the Cochran's *Q* statistic and the I^2 index were computed, such that the greater the I^2 value, the greater observed heterogeneity, with values equal to 25%, 50% and 75% reflecting low, moderate, and large heterogeneity, respectively (Huedo-Medina et al. 2006). If evidence of heterogeneity was found, the influence of moderator variables was analyzed by

assuming mixed-effects models for each bacteria analyzed with at least 7 effect sizes. Weighted ANOVAs and meta-regressions were performed to test the influence of the potential categorical and continuous moderators on the effect sizes, respectively. The improved method proposed by Knapp and Hartung (2003) was applied to test the statistical significance of each moderator variable (Rubio-Aparicio et al. 2019). Q_W and Q_E statistics were computed to assess model misspecification for weighted ANOVAs and meta-regressions, respectively, and an estimate of the proportion of variance accounted for by the moderator variable (R^2) was calculated (López-López et al. 2014). Publication bias was assessed by constructing funnel plots with the trim-and-fill method (Duval and Tweedie 2000) and by applying Egger´s regression test (Rothstein et al. 2005). These analyses were performed for each bacteria analyzed with at least 7 effect sizes, as these techniques are not reliable with smaller data sets.

All statistical analyses were carried out with the *metafor* package in *R* (Viechtbauer 2010).

Re-analyzing previous meta-analyses

To compare Iglesias-Vázquez et al. (2020) and Xu et al. (2019) results to ours, their data had to be re-analyzed by calculating an effect size index similar to the one we used, and by applying synthesis methods similar to those applied in our meta-analysis. Considering the forest plots presented in Iglesias-Vázquez et al. (2020), we calculated the difference between the average relative abundance of ASD and control groups (D) for each primary study, and its variance was estimated by means of the following equation: $V(D) = (SE_{ASD})^2 + (SE_{Control})^2$, with SE_{ASD} and $SE_{Control}$ being the standard errors for the ASD and Control groups, respectively, reported in the forest plots. Then, the probability level associated with the statistical significance of the overall difference

between relative abundances of the studies (D_+) was compared with the probability level of the statistical significance of the overall standardized mean difference (SMD_+) obtained in our meta-analysis. It is important to note that our purpose was not to re-analyze all the results found in Iglesias-Vázquez et al.'s (2020) and Xu et al.'s (2019) studies, but only those of phyla and genera included in our meta-analysis, with the aim of allowing a comparison of their results with ours. In particular, for Iglesias-Vázquez et al.'s (2020) meta-analysis, we re-analyzed the results for phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, and for genera *Bacteroides*, *Clostridium*, *Roseburia*, *Ruminococcus*, *Suterella*, and *Bifidobacterium*. For Xu et al.´s (2019) meta-analysis, re-analyses were accomplished for the genera *Ruminococcus* and *Clostridium* only. For genera *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* it was not needed to recalculate the effect sizes because Xu et al. (2019) applied just the same effect size index and synthesis methods than ours.

Results

Characteristics of the included studies

Three authors formed the review team in order to implement measures to minimize errors and bias at all review stages and independently screen titles, abstracts and full texts of the works for potential inclusion. Two reviewers evaluated them according to the eligibility criteria. Disagreements on whether a given reference should be included or not were resolved through discussion with a third reviewer.

The 2391 studies identified from the four databases were crossed with the EndNote X7 software to detect the possible duplicates. After reviewing the abstract of each of the remaining articles, those that were related to the subject of the study were initially selected as eligible studies. The complete articles were downloaded from different

webpages. Data from 14 articles were requested by email from the authors, receiving only one reply. Finally, Figure 1 shows that a total of 18 articles were selected for the present systematic review and meta-analysis following the full-text eligibility assessment.

INSERT FIGURE 1

The characteristics and main findings of the 18 selected articles in the present systematic review and meta-analysis are summarized in Supplementary Table 2. The 18 selected articles (Parracho et al. 2005; Finegold et al. 2010; Adams et al. 2011; Williams et al. 2011; Wang et al. 2011; Williams et al. 2012; Wang et al. 2013; De Angelis et al. 2013; Kang et al. 2013; Inoue et al. 2016; Iovene et al. 2017; Finegold et al. 2017; Kang et al. 2018; Coretti et al. 2018; Zhang et al. 2018; Ma et al. 2019; Plaza-Díaz et al. 2019; Niu et al. 2019) were published between 2005 and 2019. According to the corresponding author addresses, the articles were published in 7 different countries: seven in the USA, three in Italy, three in China, two in Australia, and one in Spain, UK and Japan.

The 18 articles selected analyzed a total of 998 participants, with 642 pertaining to ASD groups (range: 6 - 114, mean = 36) and 356 to the control groups (range: 6 - 57, mean = 20). Ten studies (55.5%) showed a medium-high quality on the NOS scale, and 14 (77.7%) did not present reporting bias (i.e., only reporting data for statistically significant variables). On the other hand, 66.6% of the studies exhibited a good balance between ASD and control sample sizes (see Supplementary Table S3).

Mean effect size and heterogeneity

Main meta-analytic results regarding the mean standardized mean difference estimates and heterogeneity are presented separately for bacterial phylum and bacterial genus.

Bacterial Phyla

Table 1 presents the results of the overall standardized mean difference in bacterial abundance of children with ASD compared to control groups for *Bacteroidetes, Firmicutes, Proteobacteria,* and *Actinobacteria* phyla.

The largest mean effect size, computed through 6 studies, was found for *Firmicutes* (SMD_+ = -0.484), which can be considered low-magnitude and not statistically significant. The negative sign indicated that the presence of *Firmicutes* in gut microbiota was slightly lower in ASD groups than in control groups. For *Actinobacteria* (k = 6), the mean effect size was also negative, but with a low magnitude and not statistically significant (SMD_+ = -0.317). A positive mean effect size, with a low magnitude and statistical insignificance, was found for *Bacteroidetes* (SMD_+ = 0.224), yielding a slightly larger presence of this bacterial phylum in children with ASD than in control groups. For *Proteobacteria,* the overall effect size was practically null (SMD_+ = 0.053). Supplementary material contains the forest plots constructed for *Bacteroidetes, Firmicutes, Proteobacteria* and *Actinobacteria* phyla (see Figures S1, S4, S11 and S13, respectively). A large heterogeneity among the standardized mean differences for these four bacterial phyla was found (I^2 > 75% and p < .05, in all cases) (see Table 1).

INSERT TABLE 1

Bacterial Genera

Table 1 also shows the results of the overall standardized mean difference in bacterial abundance in children with ASD compared to control groups for *Bacteroides,*

Prevotella, Lachnospira, Ruminococcus, Clostridium, Lactobacillus, Streptococcus, Roseburia, Sutterella and *Bifidobacterium* genera.

Statistically significant overall effect sizes were found only for *Streptococcus* (SMD_+ = -0.999) and *Bifidobacterium* (SMD_+ = -0.513), in both cases the mean abundance being lower in ASD groups than in control groups. The overall effect size for *Streptococcus* yielded the largest magnitude. Negative mean effect sizes, although not statistically significant, were also found for *Prevotella* (SMD_+ = -0.660), *Lachnospira* (SMD_+ = -0.294), *Ruminococcus* (SMD_+ = -0.149), and *Roseburia* (SMD_+ = -0.085), with the largest magnitude found for *Prevotella*, and a practically null effect for *Roseburia*.

For the meta-analyses carried out on *Bacteroides, Clostridium, Lactobacillus* and *Sutterella*, the positive mean effect sizes indicated a slightly greater mean abundance in children with ASD. Specifically, the mean effect sizes for *Bacteroides* and *Sutterella* reflected small magnitudes (SMD_+ = 0.231 and SMD_+ = 0.174, respectively), and *Clostridium* and *Lactobacillus* yielded practically null effects (SMD_+ = 0.016 and SMD_+ = 0.088, respectively). Again, none of these mean effect sizes reached statistical significance. Supplementary material contains the forest plots constructed for *Bacteroides, Prevotella, Lachnospira, Ruminococcus, Clostridium, Lactobacillus, Streptococcus, Roseburia, Sutterella* and *Bifidobacterium* genera (see Figures S2, S3, S5, S6, S7, S8, S9, S10, S12, and S14, respectively).

Standardized mean differences for most of the bacterial genera analyzed presented great heterogeneity, with Q statistics reaching statistical significance and the I^2 indices above 75%. The only exception was for *Streptococcus*, which yielded a non-significant trend for heterogeneity ($Q(4) = 4.358$, $p = .359$; $I^2 = 21.52$).

Analysis of moderator variables

These analyses were applied only to bacterial phyla and bacterial genera with at least 7 effect sizes, i.e., *Bacteroidetes, Bacteroides, Proteobacteria*, and *Bifidobacterium.* Table 2 shows the results of the simple meta-regressions applied to continuous moderator variables for *Bacteroidetes, Bacteroides, Proteobacteria* and *Bifidobacterium.* The sample size of ASD was the only continuous moderator that exhibited a statistically significant relationship with the effect sizes for *Bacteroides* genus ($p < .001$) and *Proteobacteria* phylum ($p = .033$), with large percentages of variance accounted for. In particular, a negative relationship was found in both cases, such that the larger the sample size, the lower the standardized mean difference estimates for these bacteria. However, it is worth noting that the percentage of women in ASD groups for *Bacteroidetes* phylum and the percentage of women in HC groups for *Bifidobacterium* genus showed marginally statistically significant results ($p = .051$ and $p = .094$, respectively), as well as large percentages of explained variance. In addition, another continuous moderator that yielded a marginally significant relationship with the effect sizes for *Bifidobacterium* genus was the total score for the NOS ($p = .098$; $R^2 = .69$), indicating that the larger the NOS score, the lower the effect size.

INSERT TABLE 2

Supplementary Table 4 presents weighted ANOVAs of categorical moderator variables for *Bacteroidetes, Bacteroides, Proteobacteria* and *Bifidobacterium*. Of the different categorical moderators analyzed, only the severity level of ASD showed a statistically significant result ($p = .017$) for the *Bacteroides* genus, with a higher mean effect size for moderate-severe level of ASD than for moderate severity. Nevertheless, this result must

be interpreted cautiously due to the small number of studies in the two moderator categories.

Results of the ANOVAs applied to the items of the NOS for *Bacteroidetes, Bacteroides, Proteobacteria* and *Bifidobacterium* are presented in Supplementary Table 5. None of the items reached a statistically significant relationship with the standardized mean differences.

Analysis of publication bias

For *Bacteroidetes, Bacteroides, Proteobacteria* and *Bifidobacterium*, publication bias was assessed through Egger tests and funnel plots, applying the trim-and-fill method. In the case of the rest of the bacterial phyla and genera, this was not possible due to the small number of studies (less than 7).

Non-significant results were obtained with the Egger test for *Bacteroidetes* ($t(6) = 1.051$, $p = .334$) and *Bifidobacterium* ($t(5) = -0.599$, $p = .575$), and evidence of publication bias was found for *Bacteroides* ($t(5) = 2.098$, $p = .090$) and *Proteobacteria* ($t(5) = 2.278$, $p = .072$). Supplementary material presents the funnel plots obtained for *Bacteroidetes, Bacteroides, Proteobacteria* and *Bifidobacterium* (see Figures from S15 to S18). Applying the trim-and-fill method (which involves imputing missing effect sizes to achieve symmetry in the funnel plot where necessary), no standardized mean differences had to be imputed for *Bacteroidetes* (see Figure S15) or *Proteobacteria* (see Figure S17). For *Bacteroides*, two additional effect sizes were imputed to the original set of estimates to achieve symmetry in the funnel plot (see Figure S16). When a mean effect (and its 95% CI) was calculated using the 7 effect sizes plus the two imputed values, the average effect was $SMD_{+} = 0.049$ (95% CI = -0.441, 0.541). For *Bifidobacterium*, an additional standardized mean difference estimate was imputed to the original set of estimates to avoid asymmetry in the funnel plot (see Figure S18). The

new mean effect size (and its 95% CI) computed through the 7 effect sizes plus the imputed value was SMD_{+} = -0.439 (95% CI = -0.792, -0.087). Although the publication bias-corrected overall estimate was slightly lower than the original one (SMD_{+} = -0.439 and -0.513), the corrected overall effect size retained statistical significance.

Re-analysis of previous meta-analyses

Table 3 presents the original results reported in Iglesias-Vázquez et al's. (2020) meta-analysis and those obtained by calculating the difference between the average relative abundance of ASD and Control groups from each study (*D* index). As our re-calculations were meant to make comparison possible between our results and those of Iglesias-Vázquez et al. (2020), Table 3 only reports results for the phyla and genera that were analyzed in both meta-analyses. As can be seen, Iglesias-Vázquez et al.'s (2020) results change dramatically as a function of how the results of the primary studies were statistically integrated. In particular, when a synthesis method similar to that used in our meta-analysis was applied, none of the phyla re-analyzed here (*Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*) or genera (*Bacteroides*, *Clostridium*, *Roseburia*, *Ruminococcus*, *Sutterella*, and *Bifidobacterium*) showed a statistically significant relationship between GM and ASD. These results clearly diverge from the original results obtained in Iglesias-Vázquez et al. (2020) regarding the *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* phyla, and regarding the *Bacteroides*, *Clostridium*, and *Bifidobacterium* genera.

INSERT TABLES 3 AND 4

Table 4 presents the results of the genera reported in Xu et al. (2019), and those re-analyzed by us in terms of the *D* index (*Ruminococcus* and *Clostridium*). Table 4 also shows the average *SMD*s obtained in Xu et al. (2019) for the genera *Bacteroides*,

Bifidobacterium, and *Lactobacillus*. Note that the average SMDs reported in Table 4 for *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* genera were not recalculated by us, but they were reported in their meta-analysis. In addition, note that only those genera that were analyzed in our meta-analysis are presented in Table 4. Although Xu et al. (2019) concluded that there was dysbiosis in ASD groups for *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, and *Clostridium*, the results of the overall *SMD*s and *D*s did not show statistical significance.

Discussion

The present meta-analysis integrated effect sizes of 18 studies to assess the potential association between gut microbiota and ASD. Our results did not show evidence of a relevant GM-ASD association for *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* phyla. Regarding bacterial genera, only *Streptococcus* and *Bifidobacterium* exhibited a significantly lower abundance in ASD groups than in controls. Our results are inconsistent with those of Iglesias-Vázquez et al. (2020), as they found lower abundance in ASD of *Bacteroidetes*, *Firmicutes* and *Proteobacteria* phyla. In addition, Iglesias-Vázquez et al. (2020) found greater abundance of *Bacteroides* and *Clostridium* in ASD groups, and lower abundance of *Bifidobacterium*, such that only their results for *Bifidobacterium* were consistent with ours (see Table 1). However, the discrepancies between our results and those of Iglesias-Vázquez et al. (2020) can be explained mainly by the different synthesis methods and effect size indices applied, as described above. In fact, when we re-analyzed the Iglesias-Vázquez et al. (2020) data with a synthesis method and effect size index similar to that which was applied in our meta-analysis (*D*, see Table 3), their results coincided with ours, in the sense that there was an absence of evidence for GM-ASD association in the *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* phyla, as well as in the *Bacteroides* and

Clostridium genera. Standard meta-analytic methods calculate an effect size from each individual study in order to compare the average abundance of ASD group with *its own* control group. In contrast, Iglesias-Vázquez et al. (2020) and Xu et al. (2019) calculated a pooled average abundance of ASD groups across studies and compared it with a pooled average abundance of control groups. This synthesis method can lead to confounding problems known as Simpson's paradox (Borenstein et al. 2019; Rücker and Schumacher 2008).

In their meta-analysis, Xu et al. (2019) concluded that ASD groups presented lower abundance than controls of *Bacteroides* and *Bifidobacterium* genera, a larger abundance of *Ruminococcus* and *Lactobacillus* genera, and an absence of differences for *Clostridium* genera. However, none of the overall effect sizes obtained (Xu et al. 2019) for these genera reached statistical significance (see Table 2), such that they actually did not find evidence of a clear GM-ASD association for these genera. Thus, our results are consistent with those of Xu et al. (2019), with the exception of *Bifidobacterium*. For this bacterial genus, Xu et al. (2019) obtained a lower abundance in ASD but not statistically significant, whereas we obtained both a lower abundance in ASD and statistical significance. Probably, the absence of statistical significance in Xu et al. (2019) was due to a low statistical power given the low number of studies used.

The bacterial genera that seem to be more involved in dysbiosis of the GM in children with ASD are *Streptococcus* and *Bifidobacterium*. In this sense, it has been reported in an animal model that *Bifidobaterium pseudocatenulatum* CECT 7765 has a positive effect on the hypothalamic-pituitary-adrenal (HPA) axis because it lowers anxiety levels (dopamine and adrenaline) and has an anti-inflammatory effect on the GI tract (Moya-Pérez et al. 2017). *Bifidobacterium* is one of the first bacteria to colonize the intestine of neonates. Many genera of *Bifidobacterium* have been associated with a variety of health

benefits. Thus, a dysbiosis of the *Bifidobacterium* could influence in child neurodevelopment (Martínez-González and Andreo-Martínez 2020b).

Regarding the clearly lower abundance of *Streptococcus* in children with ASD, it is known that *Streptococcus* produces lactate (Zhang et al. 2018) and serotonin (Wu et al. 2020). However, study results are difficult to interpret because high levels of lactate have been reported in children with ASD (Oh et al. 2020; Rossignol and Frye 2012). The evidence supports that there is a mitochondrial dysfunction is associated with ASD (Rossignol and Frye 2012), suggesting an increase in glycolysis through the phenomenon of aerobic glycolysis in ASD, since the dysregulation of this balance has been proposed as a potential cause of ASD (Vallée and Vallée 2018). A possible explanation for the high levels of lactose may be due to *Lactobacillus*, a genus that also produces lactic acid, and whose levels are high in ASD (Iglesias-Vázquez et al. 2020; Xu et al. 2019). Furthermore, it appears that very few *Streptococcus* species such as *Streptococcus thermophilus* produce lactate. In general, this genus is more associated with infection processes (e.g.: Da Silva and Winkelströter 2019). Therefore, low levels of the *Streptococcus* genus help to discard the hypothesis which suggests that streptococcus may have caused the neurodevelopmental alteration of the child with ASD. Thus, these findings deviate etiologically from the PANDAS syndrome (pediatric autoimmune neuropsychiatric disorders associated with streptococci) which has been associated with *group A streptococcal* infection (GAS) (Baj et al. 2020).

Regarding serotonin, high levels of this neurotransmitter have been associated with poorer speech development, impaired social communication and play skills, disruptive behavior, self-injury and GI symptoms in children with ASD (Bridgemohan et al. 2019). However, to the best of the author's knowledge, there are no studies linking the lower

abundance of *Streptococcus* with high levels of lactate and serotonin in children with ASD.

Although not statistically significant, the results of the present systematic review and meta-analysis point toward a possible higher abundance of *Bacteroides* and lower abundance of *Lachnospira* and *Prevotella* in children with ASD. In this sense, a positive correlation was also found between *Bacteroides* and free amino-acids, propionic acid and NH_3 (De Angelis et al. 2013), which are acids involved in the microbiota-gut-brain axis (Ding et al. 2017). *Lachnospira*, together with other commensals belonging to the *Lachnospiraceae* family, is a butyrate producer (Haas and Blanchard 2017) and, although a direct relationship between *Lachnospira* and neurotransmitters has not yet been evidenced, studies with populations that present anxiety showed lower abundances of this bacterial genus (Jiang et al. 2018). *Prevotella* can also produce SCFA in the GI tract from microbial exopolysaccharides synthesized by *Bifidobacterium* (Kang et al. 2013). Therefore, lower abundance of *Prevotella* and *Lachnospira* can modify SCFA level, which can induce effects on the GI tract, brain and behavior (Andreo-Martínez et al. 2019).

A large number of moderator variables were analyzed for their potential influence on the effect sizes of *Bacteroidetes*, *Bacteroides*, *Proteobacteria*, and *Bifidobacterium*. Only the sample size exhibited a statistical association with the effect sizes for *Bacteroides* genus and *Proteobacteria* phylum. These results coincide with the evidence of publication bias found for this genus and phylum.

One of the conclusions is that future intervention studies with probiotics in children with ASD should consider implementing the bacterial genera *Streptococcus* and *Bifidobacterium*. The administration of probiotics can improve some behavioral

symptoms associated with ASD, as they can stabilize the mucosal barrier by increasing mucin expression, reducing bacterial overgrowth, stimulating mucosal immunity, and synthesizing antioxidant substances (Shaaban et al. 2017). In this regard, it is noteworthy to mention that a limited number of studies published to date included *Bifidobacterium* as a probiotic (Shaaban et al. 2017; Martínez-González and Andreo-Martínez 2020a), while some species belonging to the bacterial genus *Lactobacillus* have been used traditionally as probiotics for children with ASD (Shaaban et al. 2017; Martínez-González and Andreo-Martínez 2020a). The results of the probiotic interventions in children with ASD are not conclusive. While some studies find statistical differences after the application of probiotics in emotional symptoms and symptoms in ASD, others find no such differences. Few studies have found statistical differences and have used *Bifidobacterium infantis, Lactobacillus plantarum*, or a combination of *Lactobacillus acidophilus, Lactobacillus rhamnosus* and *Bifidobacterium longum*. However, preliminary studies on possible dysbiosis in ASD have not been considered for the design of probiotics. Thus, only 33.33% of studies with probiotics and prebiotics had a randomized, double-blind, placebo-controlled design. This lack of methodological rigor implies considerable risk of bias (Martínez-González and Andreo-Martínez 2020a). Consequently, future studies should improve their intervention designs and consider the relevant role of *Bifidobacterium* in ASD when implementing an intervention with probiotics (Martínez-González and Andreo-Martínez 2020b).

Finally, a limitation of this meta-analysis was the low number of studies. This fact made it difficult to find statistically significant relationships. Another limitation was the absence of statistical data needed to calculate the effect sizes from the primary studies. Although corresponding authors were contacted in order to request and obtain

additional data, this strategy was not very successful. This prevented the analysis of other phyla and genera included in previous meta-analyses (Iglesias-Vázquez et al. 2020; Xu et al. 2019). Another limitation was the missing data for some potential moderator variables. For example, it was not possible to analyze the percentage of autistic and healthy controls with gastrointestinal problems (constipation problems, diarrhea and abdominal pain may affect the prevalence of gut bacteria due to different transit/fermentation times) and comorbidity (ASD with or without intellectual disability). Although the quality of the studies was generally good, a reporting bias has been found in half of the analyzed studies. Therefore, the methodological rigor of future studies should be improved.

The behavior of children with ASD and GM dysbiosis found in this study cannot be directly related, due to the lack of evidence in the studies selected, as they showed an absence of psychometric analysis of the relationship between the severity of ASD behavioral symptoms and GM abundance. In addition, although cognitive difficulties are a determining factor in the severity of ASD symptoms, none of the studies indicated whether children with ASD presented a diagnosis of intellectual disability, as reported elsewhere (Martínez-González and Andreo-Martínez 2019a). Therefore, multicentre studies on the impact of GM on neurophysiology and behavior of children with ASD, as well as psychometric analyses of the correlation between the severity of ASD behavioral symptoms and GM profiles are needed.

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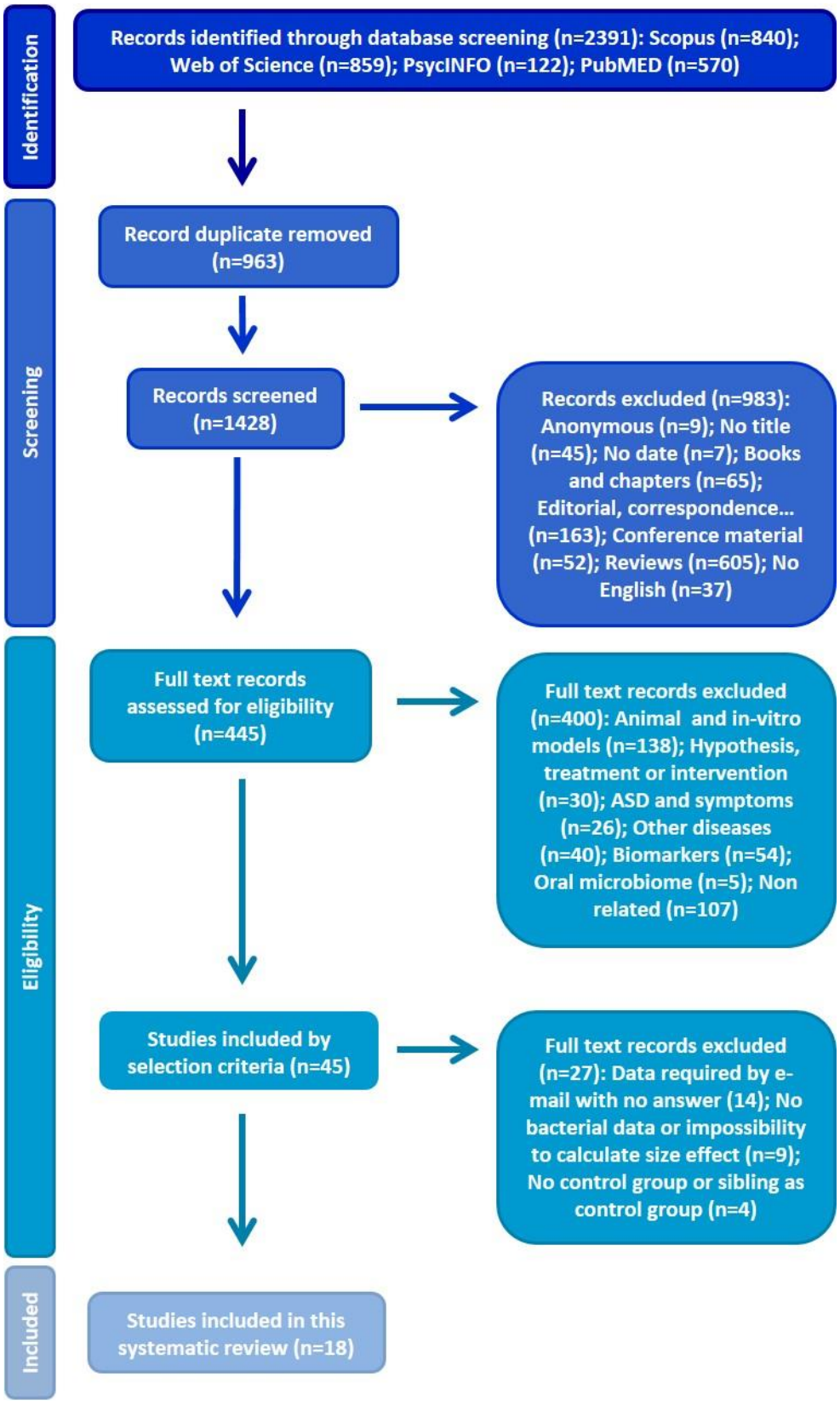

Figure 1. Flowchart showing the process of identifying relevant studies for the present systematic review and meta-analysis.

Table 1. Mean Standardized Mean Differences, 95% confidence intervals, and heterogeneity statistics at phylum and genus levels.

				95% CI			
	k	SMD_+	*p*	LL	UL	*Q*	I^2
Bacteroidetes	8	.224	.541	-.603	1.052	54.776****	90.74
Bacteroides	7	.231	.413	-.411	.874	40.829****	80.87
Prevotella	4	-.660	.166	-1.813	.492	10.441*	72.58
Firmicutes	6	-.484	.096	-1.092	.124	15.629**	75.31
Lachnospira	4	-.294	.532	-1.621	1.033	15.569**	79.14
Ruminococcus	4	-.149	.745	-1.473	1.175	14.593**	78.82
Clostridium	4	.016	.973	-1.349	1.381	20.020**	81.75
Lactobacillus	5	.088	.834	-1.007	1.184	49.334****	91.11
Streptococcus	5	-.999	.007	-1.549	-.449	4.358	21.52
Roseburia	6	-.085	.842	-1.132	.961	32.113****	87.54
Proteobateria	7	.053	.880	-.774	.879	57.768****	90.14
Sutterella	4	.174	.717	-1.212	1.559	14.238**	76.64
Actinobacteria	6	-.317	.311	-1.041	.406	33.471****	85.10
Bifidobacterium	7	-.513	.029	-.953	-.073	15.606*	62.27

k = number of studies. SMD_+ = mean standardized mean difference estimate. Positive SMD_+ indicate a larger mean abundance of bacteria in the ASD group than in the control group. *p* = probability level for the SMD_+. LL and UL: lower and upper limits of the 95% confidence interval (95% CI) for SMD_+. *Q* = Cochran's heterogeneity *Q* statistic; *Q* statistic has $k - 1$ degrees of freedom. I^2 = heterogeneity index. $* p < .05$. $** p < .01$. $**** p < .0001$.

Table 2. Results of the meta-regressions applied of continuous moderators on the Standardized Mean Differences for Bacteroidetes, Bacteroides, Proteobacteria, and Bifidobacterium.

Moderator	*k*	b_j	*F*	*p*	Q_E	R^2
Bacteroidetes						
Sample size ASD	8	-0.012	1.22	.312	30.04****	.08
Mean age ASD	6	-0.012	0.00	.959	30.64****	0
% of women ASD	7	0.100	6.52	.051	43.53****	.48
Sample size HC	8	-0.020	1.23	.309	44.53****	.06
Mean age HC	7	-0.151	1.38	.293	48.02****	.02
% of women HC	7	0.013	0.25	.636	48.96****	0
NOS score	8	0.063	0.09	.777	53.53****	0
Publication year	8	-0.037	0.12	.739	48.16****	0
Bacteroides						
Sample size ASD	7	-0.018	115.32	.000	1696.7	.99
Mean age ASD	5	0.082	0.35	.597	21.75****	0
% of women ASD	6	0.015	0.49	.521	38.39****	0
Sample size HC	7	-0.023	2.01	.216	32.48****	0
Mean age HC	5	0.111	0.43	.558	19.12**	0
% of women HC	5	-0.028	1.82	.271	13.61**	.24
NOS score	7	-0.011	0.00	.961	38.92****	0
Publication year	7	-0.001	0.00	.991	40.07****	0
Proteobacteria						
Sample size ASD	7	-0.019	8.59	.033	15.15**	.67
Mean age ASD	5	-0.198	0.52	.525	45.66****	0
% of women ASD	6	0.019	0.12	.744	55.41****	0
Sample size HC	7	-0.023	1.83	.234	53.61****	.10
Mean age HC	6	-0.167	1.48	.291	52.49****	.07
% of women HC	6	-0.007	0.07	.807	41.61****	0
NOS score	7	-0.024	0.01	.909	55.00****	0
Publication year	7	-0.079	0.72	.434	50.78****	0
Bifidobacterium						
Sample size ASD	7	-0.000	0.00	.985	14.04*	0
Mean age ASD	5	-0.043	0.08	.793	13.77**	0
% of women ASD	6	-0.011	0.39	.567	14.49**	0
Sample size HC	7	0.006	0.21	.669	15.24**	0
Mean age HC	5	-0.051	0.11	.759	13.90**	0
% of women HC	5	-0.020	5.89	.094	4.47	.91
NOS score	7	0.243	4.12	.098	7.55	.69
Publication year	7	-0.012	0.08	.784	15.57**	0

k = number of studies. b_j = regression coefficient. *F* = *F* statistic to test the statistical significance of the moderator. *p* = probability level for the *F* statistic. Q_E = Statistic for testing the model misspecification. R^2 = proportion of variance accounted for by the moderator. ASD = Autism Spectrum Disorder. HC = Healthy control. NOS score = total score obtained in the Newcastle-Ottawa Scale to assess the study quality. $*$ $p < .05$. $**$ $p < .01$. $****$ $p < .0001$.

Table 3. Original and re-analyzed results of Iglesias-Vázquez et al. (2020) meta-analysis.

		Original analyses			Data re-analyzed			
	k	ASD	Control	*p*	D_+	95%CI		*p*
Bacteroidetes	12	14.33%	10.97%	**.002**	0.82%	-1.37	3.01	.428
Bacteroides	12	9.04%	4.69%	**< .001**	0.34%	-2.11	2.78	.767
Firmicutes	11	13.42%	10.77%	**< .001**	-0.07%	-0.22	0.08	.298
Clostridium	10	0.74%	0.16%	**< .001**	0.06%	-0.11	0.24	.421
Roseburia	7	0.11%	0.09%	.630	0.01%	-0.08	0.11	.713
Ruminococcus	11	2.90%	2.21%	.170	0.52%	-0.55	1.60	.305
Proteobacteria	11	0.09%	0.02%	**< .001**	0.14%	-0.12	0.39	.255
Sutterella	7	0.11%	0.22%	.480	0.33%	-0.67	1.33	.452
Actinobacteria	11	0.53%	0.43%	.360	-0.42%	-2.38	1.55	.646
Bifidobacterium	12	0.46%	0.89%	**< .001**	-1.03%	-0.22	0.72	.223

k = number of studies. ASD and Control = overall relative abundance (%) reported in Iglesias-Vázquez et al. (2020, Table 2). D_+ = overall difference between relative abundance (%) of ASD and Control groups. 95%CI = lower and upper confidence limits around D_+.

Table 4. Original and re-analyzed results of Xu et al. (2018) meta-analysis.

	k	SMD_+	95%CI	*p*
Bacteroides	3	-0.35	-1.20 0.51	.427
Bifidobacterium	4	-1.05	-2.27 0.18	.093
Lactobacillus	3	0.53	-0.001 1.1	.059
	k	D_+	95%CI	*p*
Ruminococcus	5	-0.14%	-2.00 2.00	.839
Clostridium	4	0.27%	-4.00 4.00	.851

k = number of studies. SMD_+ = overall standardized mean differences reported in Xu et al. (2018, Figures 5A and 5B). D_+ = overall difference between relative abundance (%) of ASD and Control groups. 95%CI = lower and upper confidence limits around SMD_+ or D_+. *p* = statistical significance of SMD_+ or D_+. Results for Ruminococcus and Clostridium were re-analyzed. Results for Bacteroides, Bifidobacterium, and Lactobacillus are those reported in Xu et al. (2019).