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Lactobacilli Profile in Faecal Samples of Tunisian Children Diagnosed with Autism Spectrum Disorder

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Abstract: Alterations in faecal lactobacilli in autistic children have been reported, but little is known related to age and disorder severity. We used a culture-based method and partial 16S rRNA gene sequencing to isolate and identify lactobacilli strains from faeces of Tunisian autistic children (ASD group) and compared them with strains isolated from siblings (SIB) and children from the general population (GP). The ASD group displayed an increased number of different species compared to SIB and GP. Differences in species abundance with age accounted for a significant decrease in the abundance of *Lactiplantibacillus plantarum/Lactiplantibacillus pentosus* isolates in the GP at the age of 8–10 years compared to the age of 4–7 years, and to a significantly lower abundance of *Lacticaseibacillus rhamnosus* in the ASD group with respect to SIB and the GP at the age of 8–10 years. Simpson's and Shannon–Wiener indices showed a more pronounced species diversity increase with age in the GP group compared to the ASD and SIB groups. Minor differences were found in lactobacilli prevalence and in species diversity between children with severe and mild-to-moderate ASD. Overall, we found substantial differences in the profile of faecal lactobacilli species in the ASD and GP groups at the age of 8–10 years.

Keywords: autism; children; faeces; gut microbiota; lactobacilli; prevalence

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1. Introduction

Autism spectrum disorder (ASD) is an early-onset neurodevelopmental pathology characterised by impaired social communication and interaction and repetitive and stereotyped behaviours or interests [1]. While the aetiology of ASD remains largely unknown, a strong body of evidence revealed that alterations in the composition of the gut microbiota are associated with the development of autism [2].

Several research studies have analysed the intestinal microbiota of autistic children using culture-dependent methods as well as high-throughput DNA sequencing techniques [3–5]. An imbalance in the relative abundance of Bacteroidota (formerly named as Bacteroidetes) and Bacillota (formerly named as Firmicutes) phyla has been found, with a decrease in the Bacteroidota/Bacillota ratio in faecal samples of children with ASD [6]. A higher relative abundance of *Collinsella*, *Corynebacterium* and *Dorea* genera and of the lactobacilli group have been reported in faeces from ASD children compared to those from controls [7]. Other authors found reduced levels of *Bifidobacterium*, *Prevotella*, and *Akkermansia municiphila* in the gut microbiota of autistic children [8–10]. To date, different

and sometimes contradictory results have been reported in studies conducted on the gut microbiota composition in ASD, which can be partly attributed to the heterogeneity of some factors that can influence the microbiota such as dietary and social habits, environment, and ethnicity [11].

Lactobacilli are Gram-positive, non-sporulating, catalase-negative and fermentative group of lactic acid bacteria comprising different closely related genera [12]. These microorganisms belong to the phylum Bacillota and some members of this group are found in the human and animal gut microbiota. Lactobacilli can be clustered into two major metabolic groups based on the hexose catabolic pathway: (i) homofermentative, which metabolise hexoses via the Embden–Meyerhoff pathway to pyruvate as the main metabolic intermediate and lactic acid as the main end product, and (ii) heterofermentative, displaying a metabolism of hexoses via the phosphoketolase pathway to be converted into pyruvate and acetyl-phosphate as key intermediates, and lactic acid, acetic acid and/or ethanol as end products [12,13].

Most of the studies focusing on ASD microbiota have found alterations in the lactobacilli population when comparing the ASD group with the general population (GP). Some of them have reported high levels of lactobacilli in the faeces of ASD children [3,6,7,11], whereas De Angelis et al. found reduced levels of lactobacilli in ASD with respect to children from the control group [4]. These findings, although apparently contradictory, point towards a possible association of lactobacilli with the development of autism.

Ligilactobacillus ruminis was the most frequently isolated and predominant species found in the gut microbiota of Indian ASD children [11], whereas no identification at the species level within the lactobacilli group was carried out in other studies with different human populations within the lactobacilli group [3,4,6,7]. Indeed, the identity of lactobacilli isolated from the faeces of children with ASD is still poorly known. To our knowledge, the prevalence, species diversity, and characteristics of lactobacilli isolated from the gut microbiota of Tunisian individuals with ASD have not been previously explored. Our aim in the present work was to unveil differences in faecal lactobacilli composition among Tunisian children with ASD, their siblings and a Tunisian children's control group (ASD, SIB and GP groups, respectively), along with the putative effects of age and the severity of the disorder. To those ends, we isolated and identified the predominant lactobacilli strains from faecal samples and we compared the profiles obtained among the three groups of children. To our knowledge, this is the first report analysing differences in the profile of faecal lactobacilli in ASD in Tunisia and one of the few works in the literature considering the influences of age and disorder severity on the faecal microbiota composition in autism.

2. Materials and Methods

2.1. Subjects and Faecal Sample Collection

Faecal samples were collected from 74 Tunisian children aged between 4 and 10 years. The children were divided into three groups: 28 ASD (22 boys and 6 girls; with a mean age of 7.93 $\pm$ 2.05 years), 18 age-matched SIB (brothers and sisters of autistic children, 5 boys and 13 girls; with a mean age of 7.56 $\pm$ 1.89 years), and 28 age- and sex-matched unrelated GP children (22 boys and 6 girls; with a mean age of 7.29 $\pm$ 2.09 years). Autistic children were recruited at the Child and Adolescent Psychiatry Unit, Department of Psychiatry, Fattouma Bourguiba University Hospital, Monastir, Tunisia. The children from the general population were recruited from kindergartens and/or schools from the same social group. An ASD diagnosis was carried out by qualified personnel according to the Diagnostic and Statistical Manual for Mental Disorders (DSM-5) [14], the Autism Diagnostic Inventory-Revised (ADI-R) [15], and the Autism Diagnostic Observation Schedule-2 (ADOS-2) [16]. The severity of ASD was assessed using the Childhood Autism Rating Scale [17] (CARS). The exclusion criteria for all the participants were: having neurological disorders not strictly related to autism, type 1 diabetes, genetic syndromes, celiac disease, food intolerance, or inflammatory bowel disease. Subjects in this study were not treated with antibiotics or antifungals and had not taken probiotics and/or prebiotics for at least one month prior to

sampling. Demographic and clinical data of the participants were described in detail in a previous study [18]. The study was approved by the Ethics Committee of the Faculty of Medicine of Monastir (reference IORG 0009738 N°18/ OMB 0990-0279) and by the Bioethics Committee of CSIC (reference 172/2020), and informed written consent was obtained from the parents or legal guardians of each child before their inclusion in the study.

2.2. Isolation of Lactobacilli Strains and Culture Conditions

One gram of each fresh faecal sample was resuspended in 10 mL sterile distilled water and homogenised by vortexing for 5 min. Then, 10 µL of this homogenate was spread-plated on MRS agar (Biokar, Beaubois, France) and incubated under anaerobic conditions at 37 ° C for 48–72 h. Colonies of various morphologies and sizes were picked at random from plates and re-isolated again on MRS agar plates to obtain pure cultures. Pure isolated colonies were then inoculated in MRS broth, stained with the Gram method and tested for their ability to produce the enzyme catalase. Isolates suspected to be Gram-positive bacilli/cocobacilli and catalase-negative were stored in 30% glycerol (v/v) at −20 °C for further identification [19].

2.3. Identification of Lactobacilli Strains Using Partial 16S rRNA Gene Sequencing

Frozen pure isolates were thawed and cultured anaerobically on MRS broth for 24 h, centrifuged and the cell pellet was used for DNA extraction and amplification reactions. The identification of strains was carried out by partial sequence analysis of the 16S rRNA gene. Briefly, the bacterial DNA was extracted from the cell pellets of pure microbial cultures by using the Gen Elute^{TM} Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's instructions. The 16S rRNA was partially amplified using the universal primers plb 16 (5′-AGAGTTTGATCCTGGCTCAG-3′) and mlb 16 (5′-GGCTGCTGGCACGTAGTTAG-3′) [20]. The PCR reaction mixtures (25 µL) contained 1 µL of genomic DNA, 12.5 µL of 2X DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 1 µL of each forward and reverse primer (10 µM), and 9.5 µL of molecular biology-grade water (Sigma-Aldrich). The PCR conditions were as follows: one initial denaturing step at 95 °C for 5 min, 30 cycles at 94 °C for 45 s (denaturation), 55 °C for 60 s (annealing), 72 °C for 35 s (elongation), and a final extension step at 72 °C for 10 min. DNA amplification was performed in a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA), verified by electrophoresis on 1% agarose gel (Condalab, Torrejon de Ardoz, Spain) stained with Midori Green Advanced DNA stain (NIPPON Genetics Europe, Germany) and visualised under UV light by using a G box machine (SynGene, Cambridge, UK). Furthermore, the PCR products were purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich) and sequenced at Macrogen Inc. (Madrid, Spain) in an ABI3730XL automated sequencer (Applied Biosystems). The sequences were compared to those in the NCBI database using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 27 February 2023).

2.4. Prevalence and Species Diversity

The prevalence of lactobacilli isolates in stools of the different groups of children was estimated as the percentage of positive stool samples (with at least one lactobacilli colony isolated and identified) with respect to the total stool samples analysed in the group considered. Species lactobacilli abundance was calculated as the percentage of each species among the total lactobacilli isolates identified in each group of children.

The species diversity in each group of children considered in this study was calculated taking into account the number of species and the relative abundance of each species by using Simpson's (D) and the Shannon–Wiener (H) diversity indices, which emphasise evenness and richness of species, respectively.

Simpson's diversity index (D) was calculated by applying the following formula:

$$D = 1 - \frac{\sum n(n-1)}{N(N-1)}$$

The Shannon–Wiener diversity index (H) was calculated applying the formula:

$$H = -\sum \left(\frac{n}{N}\right) \ln\left(\frac{n}{N}\right)$$

where

D: Simpson's diversity index;
H: Shannon–Wiener diversity index;
n: number of colonies from each individual species;
N: total number of colonies from all species;
ln: natural logarithm;
Σ: sum of the calculations for all species.

The values of D range between 0 and 1 [21,22], and the values of H usually range between 0.5 and 5 [23]; the higher the value, the greater the diversity.

2.5. Statistical Analysis

Statistical analyses were carried out using the SPSS statistical pack version 26.0 (IBM SPSS, Inc., Chicago, IL, USA). Comparison of the prevalence of lactobacilli, species abundance and proportion of homofermentative and heterofermentative species among the ASD, SIB, and GP groups, as well as among the same groups stratified by age (4–7 years and 8–10 years) and by the severity of the disorder in the ASD group (mild-to-moderate with a CARS score of 30–36 and severe with a score of 37–60), was performed using the chi-square test ($\chi 2$) or Fisher's exact test. A comparison of specific lactobacilli species abundance between children from the two age ranges considered in this study (4–7 years and 8–10 years) within the same group (ASD, SIB and GP) was also performed using the same statistical tools. Differences were considered significant at p-values ≤ 0.05.

3. Results

3.1. Prevalence, Species Distribution and Characteristics of Lactobacilli in Stools of ASD, SIB and GP Children

A total of 50 Gram-positive and catalase-negative isolated colonies were identified as members of the lactobacilli group using partial 16S rRNA gene sequencing (Table S1). Out of these isolates, 21 were from faecal samples from ASD children (in four of these samples, two strains were obtained), 12 isolates were from SIB of the autistic children (one of these samples provided two strains) and 17 were from samples from the GP group (one of the samples had two isolated strains). In this way, we isolated lactobacilli colonies from faecal samples of 17 ASD, 11 SIB and 16 GP children (Figure 1). No statistically significant difference was found in the prevalence of lactobacilli among the faecal samples from the three groups of children ($p > 0.05$).

Partial sequencing of the 16S rRNA gene identified two strains as *Lacticaseibacillus casei/Lacticaseibacillus paracasei* and the other two as *Lactiplantibacillus plantarum/Lactiplantibacillus pentosus* due to the high genome similarities between the species *L. casei* and *L. paracasei* and between *L. plantarum* and *L. pentosus*. Therefore, in the rest of the paper, isolates identified as *L. casei/L. paracasei* or *L. paracasei* and as *L. plantarum/L. pentosus* or *L. plantarum* will be called *L. casei/L. paracasei* or *L. plantarum/L. pentosus*, respectively. Among the lactobacilli isolated from stools of the ASD group, *Ligilactobacillus salivarius* ($n = 8$; 38.1%) was the most abundant species, followed by *Lacticaseibacillus rhamnosus* ($n = 4$; 19%), *Lactobacillus gasseri* ($n = 3$; 14.3%), *L. plantarum/L. pentosus* ($n = 2$; 9.5%), *L. casei/L. paracasei* ($n = 2$; 9.5%), *Limosilactobacillus fermentum* ($n = 1$; 4.8%) and *Limosilactobacillus mucosae* ($n = 1$; 4.8%). In the SIB group, the following species were identified in decreasing order of abundance: *L. plantarum/L. pentosus* ($n = 5$; 41.7%), *L. rhamnosus* ($n = 4$; 33.3%), *L. salivarius* ($n = 1$; 8.3%), *L. casei/L. paracasei* ($n = 1$; 8.3%) and *L. gasseri* ($n = 1$; 8.3%). The isolates from the GP group were identified as *L. salivarius* ($n = 6$, 35.3%), *L. plantarum/L. pentosus* ($n = 5$, 29.4%), *L. rhamnosus* ($n = 3$; 17.6%), *L. fermentum* ($n = 2$; 11.8%) and *L. casei/L. paracasei* ($n = 1$; 5.9%) (Figure 2a). No significant difference in species abundance was found

among the three groups of children ($p > 0.05$). Nonetheless *L. plantarum/L. pentosus* were less abundant in the ASD group compared to the SIB and GP groups, *L. gasseri* was only found in faeces from the ASD and SIB groups and *L. mucosae* was only present in stools from the ASD group.

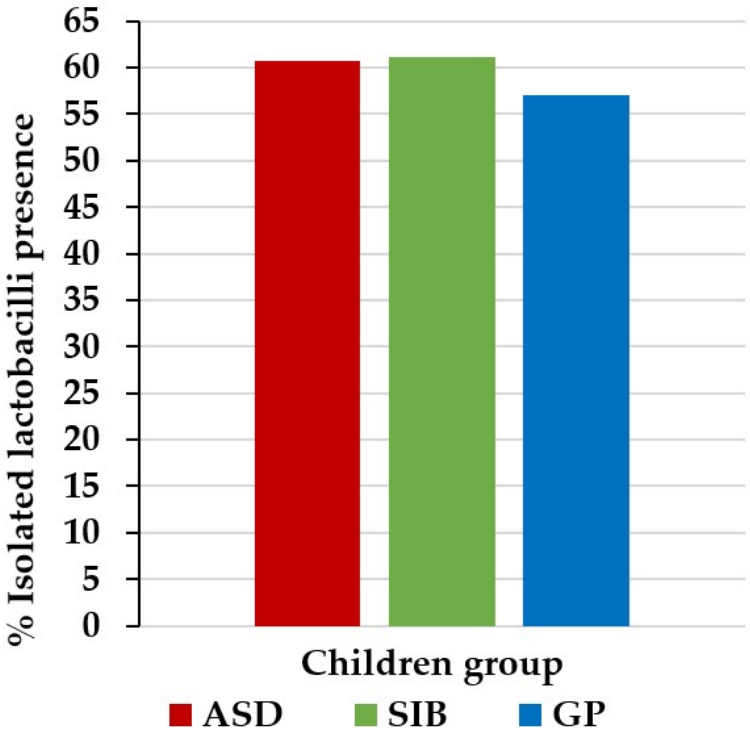

Figure 1. Prevalence of lactobacilli strains isolated from stools in autistic children (ASD), siblings (SIB) and children from the general population (GP) groups.

L. salivarius, *L. rhamnosus*, *L. plantarum*, *L. pentosus*, *L. paracasei*, *L. casei*, and *L. gasseri* are considered homofermentative species, whereas *L. fermentum* and *L. mucosae* are heterofermentative [12]. In the faecal samples from ASD children, 19 isolated colonies (90.5%) were identified as belonging to homofermentative species and only 2 isolates as heterofermentative (9.5%). In the SIB group, all isolates (100%) were identified as homofermentative species. In samples from GP children, 15 colonies (88.2%) corresponded to homofermentative microorganisms, whereas 2 colonies (11.8%) were from microorganisms belonging to heterofermentative species. When comparing proportions of homofermentative and heterofermentative species among the ASD, SIB and GP groups, no statistically significant difference was found ($p > 0.05$) among them (Table 1).

Simpson's (D) and Shannon–Wiener (H) diversity indices were determined for all species identified within each group of children, as well as for homofermentative microorganisms, providing a unique numerical value of each index for each group. The indices were not used to calculate the diversity of the heterofermentative group due to the low number of lactobacilli species considered as heterofermentative among our identified isolates. When considering all lactobacilli species, the D index was the same in the ASD, SIB and GP groups (D = 0.8), whereas the ASD and SIB groups displayed a slightly higher homofermentative species diversity (D = 0.8) than GP (D = 0.7) (Table 2). However, the H index was higher in the ASD group than in the SIB and GP groups when considering all species (H = 1.7 in ASD vs. 1.4 and 1.5 in SIB and GP groups, respectively) as well as homofermentative species (H = 1.5 in ASD vs. 1.4 and 1.2 in SIB and GP groups, respectively) (Table 2).

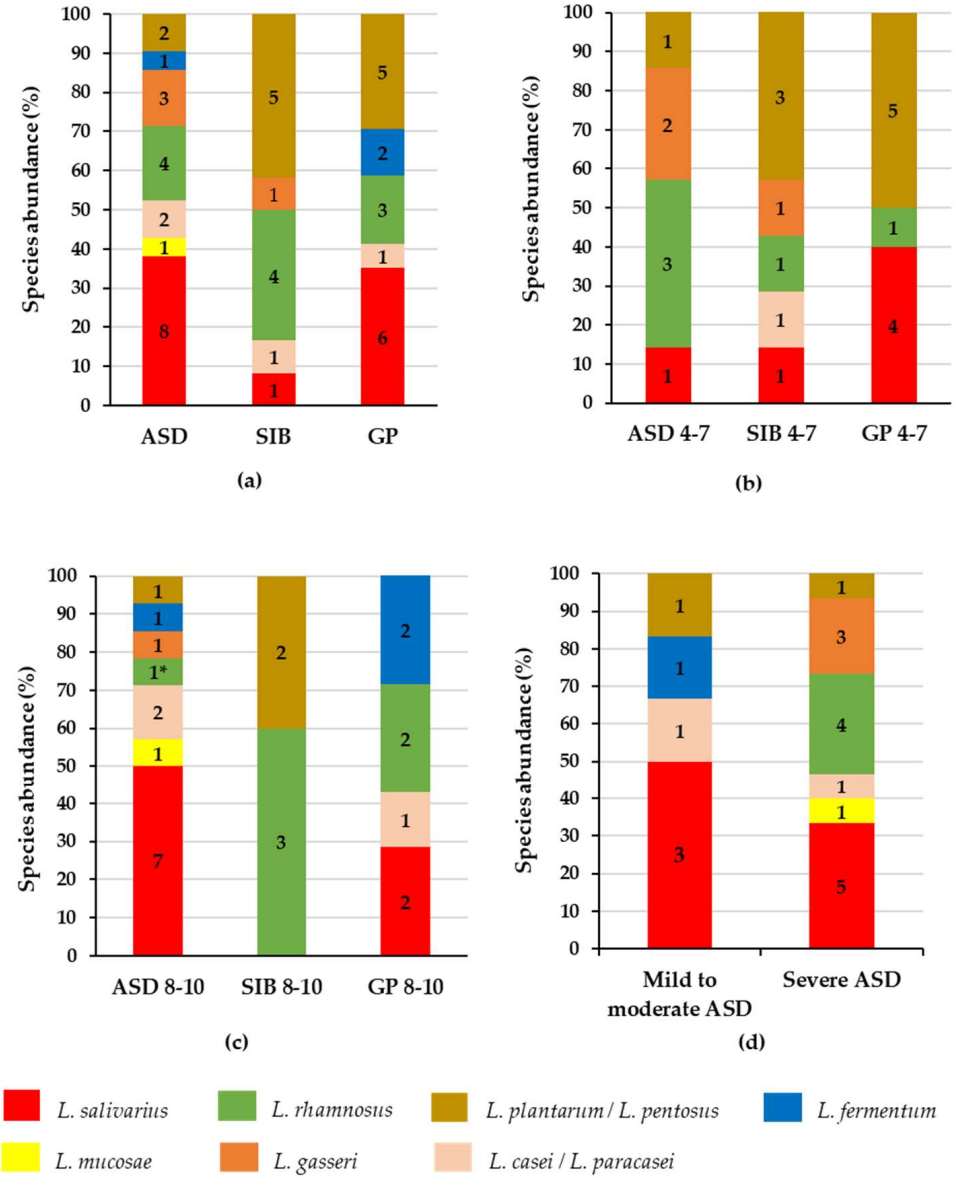

Figure 2. Species abundance of lactobacilli (%) isolated from faecal samples of autistic children (ASD), their siblings (SIB), and children from the general population (GP) considered as a whole (**a**), stratified by age—children aged between 4 and 7 years (**b**) and children aged between 8 and 10 years (**c**)—and in subgroups of ASD according to the severity of the disorder: children with mild-to-moderate ASD and children with severe ASD (**d**). The numbers inside the stacked bars indicate the absolute number of isolates from each species. Significant differences ($p \leq 0.05$) in species abundance among groups are marked with an asterisk, indicating the group with the lowest abundance.

3.2. Prevalence, Species Abundance and Diversity of Lactobacilli by Age

The prevalence, species abundance, and diversity of lactobacilli in the children's groups were also analysed separately in children aged between 4 and 7 years (n = 35, 22 boys and 13 girls; with a mean age of 5.74 ± 1.48 years) and in children aged between 8 and 10 years (n = 39, 27 boys and 12 girls; with a mean age of 9.05 ± 1.68 years). We established this sample stratification since at the age of 4 to 7 years most autistic children in Tunisia stay at home, whereas at the age of 8 to 10 years most children are assisted or are institutionalised in specialised centres.

Table 1. Proportions (%) of homofermentative and heterofermentative lactobacilli species in faecal samples from autistic children (ASD), their siblings (SIB), and children from the general population (GP) as a whole, stratified by age (4–7 years and 8–10 years) and by severity of the disorder within the group of ASD according to the CARS score (mild-to-moderate ASD and severe ASD).

		Lactobacilli Species by Fermentation Type	
		Homofermentative *n* (%)	**Heterofermentative *n* (%)**
Whole three groups	ASD	19 (90.5)	2 (9.5)
	SIB	12 (100)	0 (0)
	GP	15 (88.2)	2 (11.8)
	p-value	0.487	
4–7 years	ASD 4–7	7 (100)	0 (0)
	SIB 4–7	7 (100)	0 (0)
	GP 4–7	10 (100)	0 (0)
	p-value	-	
8–10 years	ASD 8–10	12 (85.7)	2 (14.3)
	SIB 8–10	5 (100)	0 (0)
	GP 8–10	5 (71.4)	2 (28.6)
	p-value	0.395	
ASD severity	Mild-to-moderate ASD	5 (83.3)	1 (16.7)
	Severe ASD	14 (93.3)	1 (6.7)
	p-value	0.5	

Table 2. Simpson's diversity index and Shannon–Wiener diversity index of lactobacilli species isolated from faecal samples from autistic children (ASD), their siblings (SIB), and children from the general population (GP) as a whole and stratified by age (4–7 years and 8–10 years) and from subgroups of autistic children according to the severity of the disorder (mild-to-moderate ASD and severe ASD).

		Simpson's Diversity Index		**Shannon–Wiener Diversity Index**	
		All Species	**Homofermentative Species**	**All Species**	**Homofermentative Species**
Whole three groups	ASD	0.8	0.8	1.7	1.5
	SIB	0.8	0.8	1.4	1.4
	GP	0.8	0.7	1.5	1.2
4–7 years	ASD 4–7	0.8	0.8	1.3	1.3
	SIB 4–7	0.9	0.9	1.5	1.5
	GP 4–7	0.6	0.6	0.9	0.9
8–10 years	ASD 8–10	0.8	0.7	1.6	1.2
	SIB 8–10	0.7	0.7	0.7	0.7
	GP 8–10	0.9	0.8	1.4	1.1
ASD severity	Mild-to-moderate ASD	0.8	0.7	1.2	1
	Severe ASD	0.8	0.8	1.6	1.4

In the age of 4–7 years, lactobacilli were isolated from faecal samples of 5 ASD children (50%), 6 SIB (54.5%) and 10 GP individuals (71.4%) (Table S2). The isolates from the ASD group were identified as *L. rhamnosus* (*n* = 3; 42.9%), *L. gasseri* (*n* = 2; 28.6%), *L. plantarum/L.*

pentosus (n = 1; 14.3%), and *L. salivarius* (n = 1; 14.3%). Isolates from the SIB group belonged to the species *L. plantarum/L. pentosus* (n = 3; 42.9%), *L. salivarius* (n = 1; 14.3%), *L. casei/L. paracasei* (n = 1; 14.3%), *L. gasseri* (n = 1; 14.3%) and *L. rhamnosus* (n = 1; 14.3%). Isolates from samples from the GP group were identified as *L. plantarum/L. pentosus* (n = 5, 50%), *L. salivarius* (n = 4; 40%) and *L. rhamnosus* (n = 1; 10%) (Figure 2b). Although *L. salivarius* and *L. plantarum/L. pentosus* were more abundant in GP than in the ASD group and *L. rhamnosus* was more abundant in ASD children, no significant differences in species abundance among groups of children were found at the ages of 4 to 7 years ($p > 0.05$). In this age range, all lactobacilli isolates were homofermentative species (Table 1), with a lower diversity (D and H indices) in the GP than in the ASD and SIB groups (Table 2).

In the age range of 8–10 years, lactobacilli were isolated from stools from 12 ASD (66.7%), 5 SIB (71.4%) and 6 GP children (42.9%) (Table S2). The ASD isolates were identified as *L. salivarius* (n = 7; 50%), *L. paracasei/L. casei* (n = 2; 14.3%), *L. fermentum* (n = 1; 7.1%), *L. gasseri* (n = 1; 7.1%), *L. mucosae* (n = 1; 7.1%), *L. rhamnosus* (n = 1; 7.1%) and *L. plantarum/L. pentosus* (n = 1; 7.1%). In the SIB group, the isolates were identified as *L. rhamnosus* (n = 3; 60%) and *L. plantarum/L. pentosus* (n = 2; 40%). In faeces from GP children, the isolates were identified as *L. salivarius* (n = 2; 28.6%), *L. rhamnosus* (n = 2; 28.6%), *L. fermentum* (n = 2; 28.6%) and *L. casei/L. paracasei* (n = 1; 14.3%) (Figure 2c). Remarkably, *L. rhamnosus* was significantly less abundant in ASD than in SIB and GP children at this range of age (p = 0.05). In the ASD group, 12 isolates (85.7%) were identified as homofermentative species and 2 (14.3%) as heterofermentative. In the SIB group, all isolates (n = 5; 100%) were homofermentative. In the GP group, five of the isolates (71.4%) were homofermentative and two (28.6%) were heterofermentative. However, when comparing the proportions of homofermentative and heterofermentative species at 8–10 years of age, no significant differences were found among the ASD, SIB and GP groups (Table 1). In the age range of 8–10 years, Simpson's diversity index (D) for all species as well as for homofermentative isolates was slightly higher in the GP than in the ASD group, whereas the Shannon–Wiener index (H) remained higher in ASD than in the GP and SIB groups (Table 2).

Finally, we analysed the variations in abundance of specific lactobacilli species for each group of children (ASD, SIB and GP) between the two ranges of age considered (Table 3). Despite some minor differences observed, the only significant change found in the transition from 4–7 years to 8–10 years was a decrease in the proportion of *L. plantarum/L. pentosus* (p = 0.04) in the group of GP children that did not occur in the ASD and SIB groups. As a consequence, the ratio of *L. rhamnosus* to *L. salivarius* species decreased at the age of 8–10 years in the ASD group (3 at 4–7 years vs. 0.1 at 8–10 years), whereas it increased in the GP group (0.25 at 4–7 years vs. 1 at 8–10 years). Notably, in the two ranges of age considered, we observed that Simpson's diversity index decreased while aged 8–10 in the ASD and SIB groups, while it increased in the GP group with respect to the 4–7-year age category. An increase in the Shannon–Wiener index was found in both ASD and GP groups from 4–7 years to 8–10 years, this increase being more pronounced in the GP group and coincident with a clear increase in the diversity of homofermentative species in this GP group.

In short, some differences in the distribution of lactobacilli species were found between faeces from ASD and GP children, with *L. plantarum/L. pentosus* and *L. rhamnosus* accounting for the more prominent differences observed at the ages of 8–10 years but not in younger children.

3.3. Prevalence, Species Abundance and Diversity of Lactobacilli by Severity of the Disorder

We stratified the group of ASD children into two subgroups according to the severity of the disorder following the CARS score, mild-to-moderate (n = 11) and severe (n = 17), and analysed the differences in the prevalence, abundance and diversity of lactobacilli species between these two subsets.

Table 3. Abundance of specific lactobacilli species isolated from each group of children: autistic children (ASD), siblings (SIB) and children from the general population (GP) in the two age ranges (4–7 years and 8–10 years).

Abundance		Number of Lactobacilli Isolates from ASD (n = 21)		Number of Lactobacilli Isolates from SIB (n = 12)		Number of Lactobacilli Isolates from GP (n = 17)	
		4–7 y (*n* = 7)	8–10 y (*n* = 14)	4–7 y (*n* = 7)	8–10 y (*n* = 5)	4–7 y (*n* = 10)	8–10 y (*n* = 7)
Ligilactobacillus salivarius	Number of isolates, *n* (%)	1 (14.3)	7 (50)	1 (14.3)	0 (0)	4 (40)	2 (28.6)
	p-value	0.17		1		1	
Lacticaseibacillus rhamnosus	Number of isolates, *n* (%)	3 (42.9)	1 (7.1)	1 (14.3)	3 (60)	1 (10)	2 (28.6)
	p-value	0.09		0.22		0.54	
Lactobacillus gasseri	Number of isolates, *n* (%)	2 (28.6)	1 (7.1)	1 (14.3)	0 (0)	-	-
	p-value	0.25		1			
Lactiplantibacillus plantarum/Lactiplantibacillus pentosus	Number of isolates, *n* (%)	1 (14.3)	1 (7.1)	3 (42.9)	2 (40)	5 (50)	0 (0)
	p-value	1		1		0.04 *	
Lacticaseibacillus paracasei/Lacticaseibacillus casei	Number of isolates, *n* (%)	0 (0)	2 (14.3)	1 (14.3)	0 (0)	0 (0)	1 (14.3)
	p-value	0.53		1		0.41	
Limosilactobacillus mucosae	Number of isolates, *n* (%)	0 (0)	1 (7.1)	-	-	-	-
	p-value	1					
Limosilactobacillus fermentum	Number of isolates, *n* (%)	0 (0)	1 (7.1)	-	-	0 (0)	2 (28.6)
	p-value	1				0.15	

* Significant difference ($p \leq 0.05$).

Lactobacilli were isolated from faecal samples from 6 children with mild-to-moderate ASD (54.5%) and 11 children with severe ASD (64.7%). Out of the 21 isolates from the ASD group, 6 of them were from individuals with mild-to-moderate ASD and 15 from children with severe ASD (Table S2). In stools of children with mild-to-moderate ASD, the isolates were identified as *L. salivarius* (*n* = 3; 50%), *L. plantarum/L. pentosus* (*n* = 1; 16.7%), *L. fermentum* (*n* = 1; 16.7%) and *L. casei/L. paracasei* (*n* = 1; 16.7%). In children with severe ASD, the isolates were identified as *L. salivarius* (*n* = 5; 33.3%), *L. rhamnosus* (*n* = 4; 26.7%), *L. gasseri* (*n* = 3; 20%), *L. plantarum/L. pentosus* (*n* = 1; 6.7%), *L. mucosae* (*n* = 1; 6.7%) and *L. casei/L. paracasei* (*n* = 1; 6.7%) (Figure 2d). Out of the six isolates from children with mild-to-moderate ASD, five (83.3%) of them were homofermentative and one (16.7%) was heterofermentative. In children with severe ASD, 14 isolates (93.3%) were homofermentative and 1 (6.7%) was heterofermentative (Table 1).

Simpson's diversity index (D) for all species was the same in the two ASD subgroups, whereas the Shannon–Wiener index increased from children with mild-to-moderate to children with severe symptoms of ASD. The children with a severe degree of the disorder displayed a higher homofermentative species diversity than children with mild-to-moderate ASD on both diversity indices (Table 2).

No significant differences were found in the prevalence, species abundance and proportions of homofermentative and heterofermentative species of lactobacilli isolates from the faecal samples from the two ASD subgroups. Remarkably, some lactobacilli species such as *L. mucosae*, *L. rhamnosus* and *L. gasseri* were only isolated from faeces of children with a severe degree of the disorder.

4. Discussion

ASD is one of the most common neurodevelopmental disorders and its prevalence has increased rapidly in recent years. Several studies have demonstrated an alteration in the gut microbiota composition of autistic children as compared to typically developing individuals [2]. It has been suggested that lactobacilli, which naturally inhabit the gastrointestinal tract of humans and animals, can display altered levels in the faeces of autistic children [3,4,7]. Nonetheless, the data on the link between this group of microorganisms and autism are inconsistent to date and little is known about the prevalence, species identity and characteristics of lactobacilli in autistic children. Thus, in this study, we used a culture-

based method to isolate lactobacilli strains from the faeces of Tunisian autistic children and identified them using partial 16S rRNA gene sequencing. We aimed to determine the possible variations in the faecal lactobacilli population in Tunisian autistic children through comparisons with a group of SIB and individuals from the GP, with special focus on the effects of age and ASD severity.

4.1. Prevalence and Species Abundance of Lactobacilli in Stools of ASD, SIB and GP Groups

Although we obtained a slightly higher prevalence of lactobacilli isolates in the faeces of children with ASD and their SIB compared to the GP group, these differences did not reach statistical significance.

Among the lactobacilli species identified in our work, *L. salivarius* was the most frequently isolated from the faeces of the ASD group, followed by considerably lower proportions of other species, including *L. rhamnosus*. *L. salivarius* was also the most abundant species in the GP group, whereas *L. plantarum/L. pentosus* were the most abundant species isolated from faecal samples of the SIB group. It has been reported that *L. salivarius* can induce in vitro the secretion of proinflammatory cytokines such as TNF-α by dendritic cells [24]. Elevated levels of certain proinflammatory cytokines such as IL-1, IL-6, and IL-12p40 have been found in the plasma of autistic children and have been strongly associated with the impaired communication and aberrant behaviours observed in autism [25]. In this way, we could tentatively speculate that there is a relationship between elevated levels of *L. salivarius* in the gut and a more proinflammatory status in autistic children.

In contrast to the study of Pullikan et al. [11], we did not find the species *L. ruminis*, with recognised immunomodulatory properties, in the faeces of our autistic children. However, the differential presence of some other species between the groups of children deserves some comment. *L. gasseri* was isolated from faeces from the ASD and SIB groups but not from the faeces from the GP group. The strain *L. gasseri* NK109, isolated from a human faecal sample, has been demonstrated to reduce colitis and neuroinflammation in mice via modulation of gut microbiota, leading to the attenuation of cognitive deficits and depression as well as to the improvement of neuropsychiatric disorders [26]. *L. mucosae* was only detected by us in the faeces from ASD children, and not from the other two groups of children. Recently, it has been reported that *L. mucosae* NK41, isolated from healthy human gut microbiota, can alleviate neuropsychiatric disorders by regulating altered gut microbiota in a mouse model [27]. Thus, the species *L. gasseri* and *L. mucosae*, mainly isolated in the present work from faecal samples from ASD children and generally considered as beneficial for health, may be playing a protective and beneficial role in some way for autistic children. Although we cannot know if the balance between the different lactobacilli species may influence the symptoms of the disorder and intestinal health, our results suggest differences in the profile of lactobacilli species between autistic children and children from the general population that would need a more in-depth study.

4.2. Prevalence, Species Abundance and Diversity of Lactobacilli by Age and Disorder Severity

One of the most important factors affecting the prevalence and diversity of lactobacilli in the gut microbiota is age [28]. Hence, here we analysed the prevalence, species diversity and abundance of lactobacilli strains by stratifying our sample into two age ranges (4–7 years and 8–10 years). In this way, we found some variations in the abundance of lactobacilli species according to age. Two remarkable changes occurred in species abundance as a function of age. First, at the ages of 8–10 years, *L. rhamnosus* was significantly less abundant in ASD children than in the SIB and GP groups. Second, a significant decrease in the abundance of *L. plantarum/L. pentosus* was found in the faeces from GP children at the ages of 8–10 years compared to 4–7-year-old children, a shift that did not occur in the faecal samples from SIB and ASD children. *L. rhamnosus* is known for its immunomodulatory and anti-inflammatory effects, and many strains have been widely used as probiotics [29–31]. In contrast, the proinflammatory or modulatory effect of *L. salivarius* has been found to be strain-dependent [32]. We tentatively speculate that the balance between *L. rhamnosus* and

L. salivarius in the faeces from ASD children at more advanced ages may be related to the inflammatory status of their immune system, although this aspect deserves to be investigated in more depth. In contrast to the more pronounced differences in the lactobacilli species profiles found in older children, we recently reported in the same population that there were more pronounced differences in the profile of faecal amino acids in younger children (4 to 7 years) than in those with more advanced ages (8 to 10 years) [18].

The species diversity in the three groups of children was also influenced by age. Simpson's and Shannon–Wiener diversity indices were lower in the GP group than in the ASD and SIB groups at the ages of 4–7 years while Simpson's index was higher in the GP group than in the ASD and SIB groups at the ages of 8–10 years. The evolution of species diversity with age accounts for the increase in both indices at the ages of 8–10 years in the GP group, in contrast with the less pronounced or non-occurring increase in ASD and SIB groups. The infant's gut microbiota initially exhibits a low diversity that increases with age from infancy to adulthood. In general, a healthy gut microbiota in infants and young children is characterised by a low species diversity versus a high species diversity in adults and older healthy individuals [33]. Here, in our study, we found that GP children showed an increase in lactobacilli species diversity and homofermentative species with age, which could be related to a healthier gut microbiota in the GP group.

No significant differences were found in terms of autism severity, despite a slightly higher abundance of lactobacilli species in children with severe symptoms compared to children with mild-to-moderate autism, the presence of some species only in individuals with severe symptoms of the disorder, and higher Shannon–Wiener diversity in the group with severe ASD symptoms.

This is one of the few studies currently available about faecal lactobacilli isolates in ASD and the sole considering the influence of age on the faecal lactobacilli species composition in autistic children. The results obtained highlighted clear differences in lactobacilli species composition between autistic and non-autistic children as a function of age.

5. Conclusions

The results obtained in the present work point to the few but clear differences in the profiles of faecal lactobacilli species from ASD and GP Tunisian children as related to age. Although the low number of lactobacilli isolates precludes formulating a sound hypothesis, these preliminary results lay eyes on one of the most controversial microbial group from the gut microbiota in ASD, lactobacilli. Moreover, this work emphasises the need for further studies with a larger human sample size and the use of culturomic approaches to reveal the importance of specific microbial groups in different neurodevelopmental disorders.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres14030082/s1, Table S1: Molecular identification of lactobacilli strains isolated from stools of autistic children (ASD), their siblings (SIB) and children from the general population (GP); Table S2: Number and prevalence of lactobacilli isolates in stools from autistic children (ASD), siblings (SIB) and children from the general population (GP) stratified by age (4–7 years and 8–10 years) and according to the severity of disorder (mild-to-moderate and severe). *P*-values are indicated for statistical comparisons among the three groups of children and between the two ranges of severity of disorder for the presence or absence of lactobacilli isolates in stool samples.

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Informed Consent Statement: Written informed consent was obtained from all parents of children included in the study.

Data Availability Statement: All data generated or analysed during this study are included in this article and its supplementary information files.

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