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RESEARCH ARTICLE

An Open Label Randomized Control Trial to Assess the Impact of Ayurveda Lifestyle Guidelines and Polyherbal Compounds in Bacterial Flora W.S.R to *E. coli* and *Shigella* in Children with Autism Spectrum Disorder

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Abstract

Skyrocketed numbers of autism reports with an incremental surge in its prevalence getting multiplied by four in the past decade is wary of an epidemic afoot. A randomised control trial was conducted to evaluate the effectiveness of Ayurveda polyherbal compounds and lifestyle guidelines in addition to the conventional therapies of speech therapy, occupational therapy and behavioral therapy in test group compared to their controls in the bacterial flora for the species of *E. coli* and *Shigella* done by 16 S ribosomal RNA sequencing. A clinical trial was conducted at the Out-patient and In-patient units of Vaidyaratnam P. S. Varrier Ayurveda College wherein a total of 60 ASD diagnosed children were included with a random allocation of 1:1 as test group and control group. Test group received Ayurveda poly-herbal formulations and dietary and lifestyle guidelines for 30 days along with multidisciplinary interventions for 2 months however control group received multidisciplinary therapies along with dietary and lifestyle guidelines alone for 2 months. Significant changes were obtained as results with the species of both *E. coli* and *Shigella* which was of primordial dominance before treatment has been reduced after treatment in the test group. After one month of medication in the test group much influential positive changes has occurred whereas such significance is not noted in the control group meaning that the conventional therapies of speech therapy and occupational therapy alone could not make an alteration. Polyherbal compounds of *Rajanyadi choornam* and *Vilwadi gulika* along with the dietary lifestyle guidelines and multidisciplinary interventions helps the autistic children to reduce their relative abundance for the species of *E. coli* and *Shigella*.

Keywords: Autism, Ayurveda Lifestyle Guidelines, *E. coli*, Gut Micobiota, *Rajanyadi choornam*, *Vilwadi Gulika*, *Shigella*, 16 S r RNA Sequencing

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1. Introduction

Autism Spectrum Disorder (ASD), a neuro-behavioural disorder characterized by impairment in social interaction and communication creates much turbulence in the family which needs to be addressed dexterously[1]. Skyrocketed numbers of autism reports with an incremental surge in its prevalence getting multiplied by four in the past decade is wary of an epidemic afoot[2]. As probing on the exact patho-mechanism of ASD continues, microbial imbalance with alterations in normal commensal gut microbiota known as dysbiosis has long been implicated as a possible causative mechanism contributing to the etio-pathogenesis[3]. This hypothesis is being supported by wide reports on ASD cases harboring altered gut microbiota implied through an increased incidence of Gastro Intestinal [GI] symptoms in autism children compared to their typically developed counterparts[4]. Microbiologically, the human gut microbiome virtually explains the functions and mechanisms with recent advancements in clinical research[5]. Belonging to family *Enterobacteriaceae*, both *Shigella* and *E. coli* are closely related phenotypically sharing many common characteristics[6]. In essence with 80-90% nucleotide similarity both *E. coli* and *Shigella* are human pathogens sharing similar pathogenic mechanisms[7]. From the results of a meta-analysis on association between gut microbiota and ASD it was found lower relative abundance of *E. coli* in ASD children compared to their healthy controls[8] however another study has reported higher levels of *E. coli* in ASD children compared to their controls of typically developed children[9]. Among the major therapeutic strategies for rebalancing the intestinal microbial ecosystem, probiotics are being widely accepted managing dysbiosis[10]. Both *Rajanyadi choornam* and *Vilwadi gulika* are two paramount polyherbal compounds most popularly practiced over ages for ensuring the gut health in children. A randomised control trial was conducted to evaluate the effectiveness of Ayurveda polyherbal compounds and lifestyle guidelines in addition to the conventional therapies of speech therapy, occupational therapy and behavioral therapy in test group compared to their controls. According to Ayurveda, every disease is multi-factorial of which lifestyle is the prime reason for its productivity of which ASD is of no exception and hence a multi-model approach was being selected. The aim of this study is to analyse the variations in the faecal microbiota before and after intervention with Ayurveda drugs along with lifestyle guidelines in addition to conventional therapies for the species of *E. coli* and *Shigella* for comprehensive management plan for the maintenance of gut health in autism children.

2. Materials and Methods

2.1 Trial Design

An open-label randomized controlled clinical trial was conducted at the Out-patient and In-patient units of Vaidyaratnam P. S. Varrier Ayurveda College wherein a total of 60 ASD diagnosed children were included with a random allocation of 1:1 as test group and control group. For both test group and control group the assessment was done in three phases namely Baseline (0^{th} day), Interim (30^{th} day) and Final phase (60^{th} day), however in the test group the intervention was done for 30 days (Table 1). At the time of enrolment, primary screening was done with preliminary data collection, physical examination, eligibility screening, collection of informed consent and patient allocation.

2.2 Eligible Criteria

2.2.1 Inclusion Criteria

In this study, 60 children diagnosed with ASD (DSM-V criteria) (DSM-5 2013) of either gender in the age group ranging between three to twelve years were included and was also verified for their ethnicity to make sure that they follow similar food habits and inhabit in similar environment.

2.2.2 Exclusion Criteria

All ASD children receiving anti-epileptic medications, probiotics, long term or recent (within three months) antibiotics, or ASD medications from any disciplines including Ayurveda were excluded. Children with ASD were excluded from the study if they had any co-morbid conditions like cerebral palsy, mental retardation or any other chronic neurological or metabolic disorders

Table 1. Details of the trial schedule

STUDY PERIOD				
Time point	Enrollment	Base-line phase 0	Interim phase t_1	Final phase t_2
Screening				
Preliminary data	X			
Physical examination	X			
Eligibility screening	X			
Informed consent	X			
Allocation	X			
Intervention				
Poly-herbal formulations		X		
Parental guidelines		X	X	X
Lifestyle guidelines		X	X	X
Interdisciplinary intervention		X	X	X
Assessment				
16SrRNA sequencing		X	X	X

and those who are suffering from conditions like coma, paralysis.

2.3 Intervention

According to the group allocation as test and control, a multi-modular intervention enclosing of poly-herbal Ayurveda formulations, parental and lifestyle guidelines and interdisciplinary intervention was administered. Traditional and classical practice of Ayurveda and the most modern updated Ayurvedic Formulary of India [AFI] is a treasure trove of many poly-herbal compounds meant for both gut health maintenance and disease cure. According to AFI, *Vilwadi gulika*[11] and *Rajanyadhi choornam*[12] are two paramount formulations specifically indicated for a lot of symptomatologies pertaining to gastro intestinal system and hence were selected in this trial. Ayurveda medicines was collected from Govt approved GMP certified reputed company and the patient adherence was also ensured through diary logging, pill counting and interim phone calls.

Test group with 30 participants received the two Ayurveda poly-herbal formulations (*Rajanyadi Churna, Vilwadi Guilka*) as per Ayurveda posology principles and dietary and lifestyle guidelines for 30 days (Table 2). Speech therapy, Occupational therapy and Behavioural therapy as multidisciplinary interventions was however administered to both the test and control groups for two months. Based on Ayurveda epistemology, dietary and lifestyle modifications were imparted through a well structured and prescribed guideline leaflet and one hour duration awareness classes conducted in three different sittings in the baseline phase for the parents. Throughout the trial, a checklist was also maintained for assessing and ascertaining the patient adherence to this.

2.4 Primary and Secondary Outcome Measures

Data was collected from the parents on their visit to our institution on 0^{th} day, 30th day and 60^{th} day. Primary outcome measured was the quality and quantity of the gut microbes *E. coli* and *Shigella*. Through 16 S ribosomal RNA sequencing the relative abundance was measured along with alpha and beta diversity with Illumina HiSeq2500/Miseq to generate 0.5M reads 2x250 bp for each faecal sample of the participants. 16S rRNA illumina sequencing was done by DNA isolation and purification using QiAmp mini stool kit followed by quality check and sequence processing by QIIME

Table 2. Description of the intervention plan

Sl No.	Particulars	Intervention Group	Control Group
1	Sample Size	30	30
2	Internal Administration and duration	*1. Rajanyadi churnam* *2. Vilwadi gutika* Duration – 30 days	NIL
3	Dose	As per Ayurveda Posology drug was administered with Luke warm water thrice daily, 30 minutes before meals	NA
4	Other Interventions and duration	Speech Therapy, Occupational Therapy and Behavioral Therapy Duration – 2 months	Speech Therapy, Occupational Therapy Behavioral Therapy Duration – 2 months
5	Diet modifications	As per the food and lifestyle guidelines mentioned in Ayurveda	NA

(Quantitative Insight Into Microbiological Ecology) wherein nucleic acid sequence data was analysed and interpreted from fungal, viral, bacterial and archeal communities.

The secondary outcome assessed was any changes in these species if at all occurred in the test group compared with the control group after the treatment and thus the formulation of a comprehensive approach as a protocol of Ayurveda for autism management including Ayurveda poly-herbal formulations along with the dietary and lifestyle modifications.

Patient recruitment began in May 2016, the primary and secondary outcome measure was completed in October 2020 and the study was completed in December 2020.

2.5 Sample Size

As the study followed a completely randomized design (CRD) with 2 treatments and considering the novel nature of the trial and lack of any previous information on the error variance, the number of replications was taken as 30 for each treatment. Parents were made aware of whole study design and then informed assent form was signed by at least one parent or caregivers of the corresponding child. After that the entire 60 participants were allocated randomly through computer generated randomization (1:1) into two groups of 30 each as test and control (Figure 1).

2.6 Recruitment

During their visit to Outpatient department, the parents or caretakers of the autism children were approached with a participant information file detailing the purpose of the study including the risk and benefits of participation and all the procedures to be followed during the trial. After the screening for the inclusion and exclusion criteria was done with, a brief discussion regarding the informed assent was also given. Any withdrawal or drop outs during the trial and the reasons were also duly recorded.

2.7 Data Collection Methods

During the screening data collected was recorded in a paper-based Case Report Form (CRF) and was repeated during Baseline period, Interim period (30^{th} day) and Final phase (60^{th} day).

Care was taken in the collection and storage of stool specimens as were collected by parents into sterile containers, after being given a detailed explanation of the procedure. Without breaking the cold chain, the biological samples were strictly stored at a specific temperature of -40^0C at the Research office. Later, the specimens were transported maintaining the cold chain to the concerned genetic laboratory of Medgenome Labs, Bangalore where the isolation and sequencing techniques were performed. Participant retention and

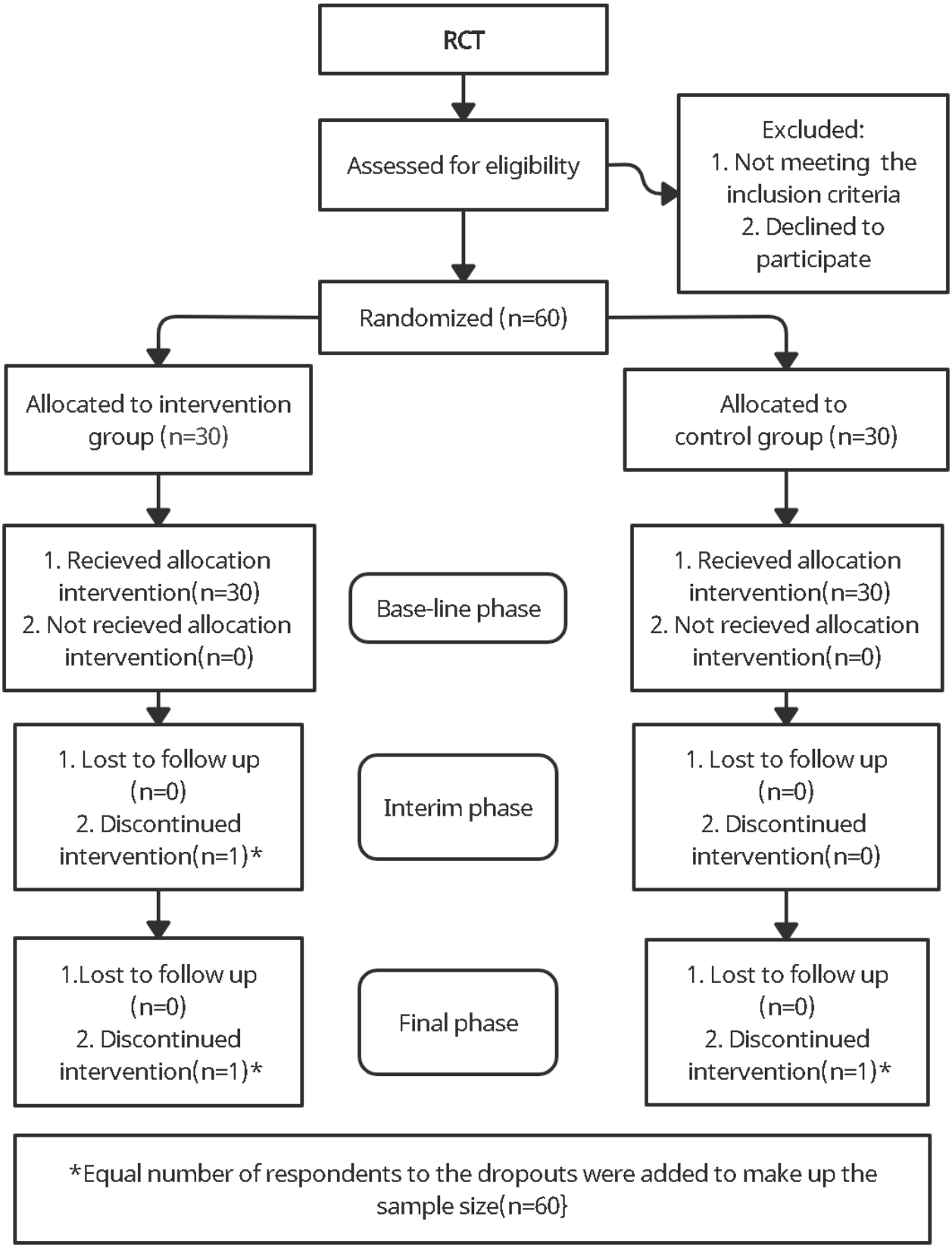

Figure 1. Study flowchart on participant allocation.

follow up was ensured even for the interdisciplinary interventions as the same was present within the same institution infrastructure.

2.8 Data Management

Paper –based CRF where the preliminary data and assessments were done was reviewed and validated by the Institutional Ethical Committee (IEC). The Data Safety Management techniques as per Indian Council of Medical Research (ICMR) guidelines were also followed for the entire trial.

2.9 Statistical Analysis

Through 16 S ribosomal RNA sequencing the microbiome taxonomic classification and sequencing was done using Operational Taxonomic Unit (OTU table, Principal Component Analysis Plot (PCA plot), Alpha and Beta Diversity. The statistical analysis for the species of *E. coli* and *Shigella* compared before and after the treatment was done using ANOVA.

2.10 Data Monitoring

For data monitoring a Data Management Committee independent from the Sponsor agency was associated for the entire trial. Any adverse events during the trial was monitored by the National Pharmacovigilance Unit of Vaidyaratnam P. S. Warrier Ayurveda College. Care was taken to maintain uniformity in ethnicity and diet practices among the selected participants.

2.11 Ethics and Dissemination

The protocol of this clinical research has undergone an initial peer-review and was approved by the IEC of Vaidyaratnam P. S. Warier Ayurveda College, India (Proceedings No: IEC/CI/24/17) on May 4, 2017. This study is a short communication extracted from a clinical trial that was registered with Clinical Trial Registry of India (CTRI) (registration No. CTRI/2018/05/014017, registered on May 21, 2018) and the entire research protocol for the entire clinical as trial is already published[13]. Any revision at any stages of the trial was submitted before the IEC for approval.

Herein this short communication is written in accordance with Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) to improve the quality of reporting[14].

2.12 Informed Consent

Parents or caregivers of the corresponding participants were made aware of the whole study design detailing every provision and the assent was obtained. Study was conducted ethically assuring the confidentiality of the participants throughout the study and with the highest respect for the individual participants in accordance with the ethical principles laid down by the *Declaration of Helsinki,* the International Conference on Harmonization Good Clinical Practice Guidelines (ICH-GCP).The Ministry of Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homoeopathy (AYUSH), Government of India will have an access to the final trial dataset as the entire trial was funded by AYUSH.

3. Observations

The distribution of volunteers in terms of gender, socio-economic status and mode of delivery are shown in Table 3, 4 and 5.

4. Results

Relative abundance of *Escherichia coli* and *Shigella* across before and after treatment calculated for significance using t-test and with FDR value less than 0.05 filtered this significant species (Figure 2).

Relative abundance of significant taxa *E. coli* before treatment and after follow-up calculated for significance using t-test and with FDR value less than 0.05 filtered this significant species (Figure 3).

Relative abundance of significant taxa *E. shigella* across before treatment and after follow-up calculated for significance using t-test and with FDR value less than 0.05 filtered this significant species (Figure 4).

5. Discussion

Escherichia coli being one of the most discussed bacterial species are having evidences on its residency in 90% healthy human microbiome though also a few

Table 3. Gender wise distribution

		Intervention	Control	
Gender	Female	08	10	18
	Male	22	20	42
Total		30	30	60

Table 4. Socio-economic status wise distribution

		Intervention	Control	
Monthly Income	less than 10, 002 (KSS-1)	02	00	02
	"10, 002-29, 972 (KSS-2) "	6	7	13
	29, 973-49, 962 (KSS-3)	10	3	13
	49, 962-74, 755 (KSS-4)	9	17	26
	99, 931-199, 862 (KSS-10)	03	03	06
Total		30	30	60

Table 5. Mode of delivery distribution

		Intervention	Control	
Mode of Delivery	Normal	22	21	43
	LSCS	8	9	17
Total		30	30	60

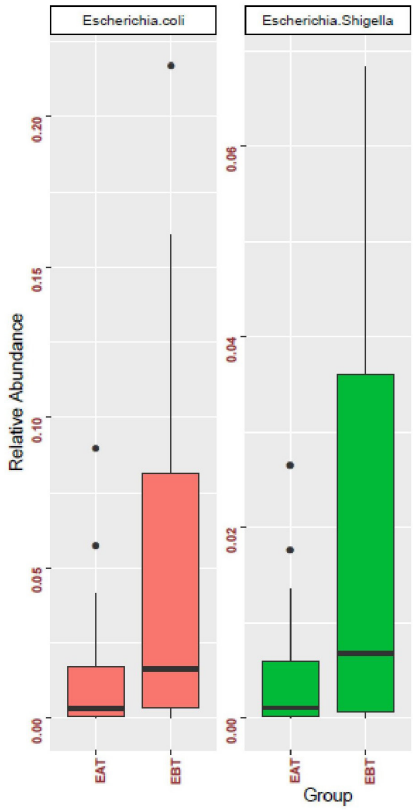

Figure 2. Relative abundance of *E. coli* and *Shigella* before and after treatment.

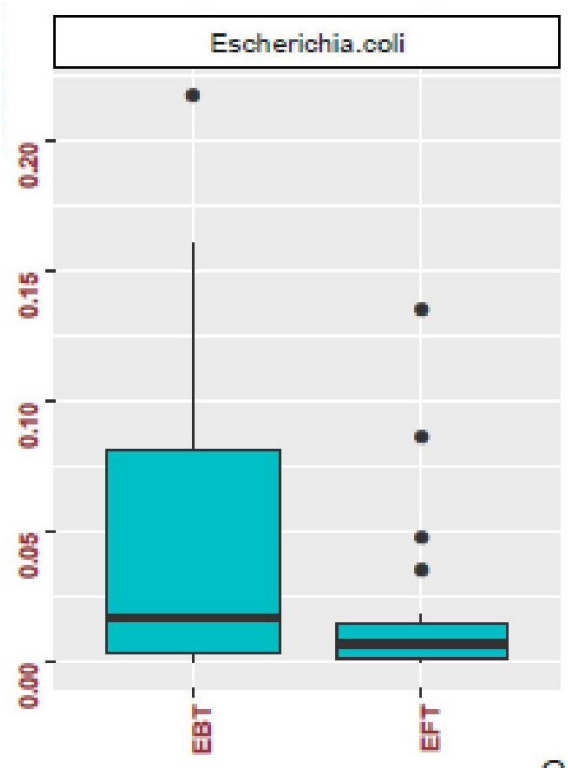

Figure 3. Relative abundance of *E. coli* before treatment and after follow-up.

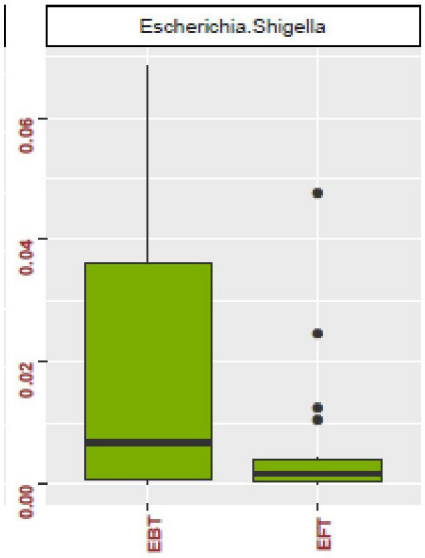

Figure 4. Relative abundance of *Shigella* before treatment and after follow-up.

present with a pathogenic ecology[15]. And genus of species of *Shigella* are gram negative bacterial pathogens most frequently isolated still posing worldwide health problems including the most devastating diarrhoea[16]. Raised *E. coli* counts in drinking water is considered major public health problem and is usually discarded regarding unsafe for human consumption[17]. From the results of community-based cross-sectional studies on sanitation mappings of ground water contamination across Kerala, despite the presence of wells with intermediate to high coliform contamination with prevalence in 73% of wells apparent rarity of water-borne diseases or associated immediate epidemic burden is reported[18]. Thus, the mere presence of *E. coli* in autistic children cannot be coupled with the morbidity be with the behavioural patterns or with the Gastro Intestinal Tract (GIT) issues. Equivocal studies are present on the relative abundance of *E. coli* in autism children as from a meta-analysis from one study it was found lower relative abundance of *E. coli* in ASD children compared to their healthy controls[8] however another study had reported higher levels of *E. coli* in ASD children compared to their controls of typically developed children. Herein with this trial on Ayurveda multidisciplinary interventions in the test group study revealed positive outcomes microbiologically from the 16 S ribosomal RNA sequencing for the species of *E. coli* and *Shigella*, nevertheless could make on a conclusive declare on this outlook. Rather appraisal on an extensive perspective enclosing the GI (Gastro Intestinal) functional analysis and autism features through Childhood Autism Rating Scale (CARS) is much essential for an unassailable come out with. Probiotic supplementation being a vital strategy in managing autism nowadays however does not bring down the morbidity as with probiotics there happens the mere replacement with beneficial bacteria and thus may cause much distortions to the naturally existing co-location of microbes. According to Ayurveda gut health being the epicenter of critical importance and thus for maintaining the homeostasis of the GI tract interventions are needed to be done in the gut itself for relieving out of such pathological conditions. In our clinical trial for the test group we had administered Ayurveda drugs of *Rajanyadi choornam* and *Vilwadi gulika* for maintaining the gut health. *Rajanyadi choornam* is a polyherbal classical Ayurveda formulation very common in the prescription for digestive impairments in children and *Vilwadi gulika* for both children and adults over years. Further assessment need to be done on whether these drugs are also acting against the abundance of *E. coli* and *Shigella*. From the results it was found that both the drugs are balancing the microbiota through the establishment of healthy equilibrium by supplying an ecosystem of different microbes. Also, from the available data from the drug review it could be found that many of the ingredient single drugs of the same are having immunomodulatory function and actions in neuro-behavioural impairments. Lifestyle and dietary guidelines have completely customized children's activities of daily life and were well reflected in the results.

Several meta analyses denotes the lower presence of *E. coli* in autistic children however there are also evidence based studies depicting the increased incidence of *E. coli* compared to the healthy controls. Any wisdom cramming on a reductionist perspective be thus defective and tend to end up with such a bipolar confusion. Be a human body or a disease or a colony of an organism or be a poly-herbal compound are not to be considered as secluded strains rather encloses the miniscule epitomes of entire universe and are beyond the linear notions and hence known as non-linear. Though at the level of microbiology highly positive

results are obtained however cannot make a conclusive note and be done only after considering the non-linear nature is being discussed here in this paper. With reduction in the relative abundance of these species after treatment in turn results in reducing autistic traits however need not be concluded as such rather may cause an increase in its abundance in some other study. Hence by merely considering a single species of microorganism gut environment and its health cannot be ascertained. Ultimate aim is the establishment of homeostasis considering collocation and interaction of species.

6. Conclusion

Polyherbal compounds of *Rajanyadi choornam* and *Vilwadi gulika* along with the dietary lifestyle guidelines and multidisciplinary interventions helps the autistic children to reduce their relative abundance for the species of *E. coli* and *Shigella*.

7. Limitations

Ethnographically though from the same area, exposure to different types of food and psychosocial exposure pluralism also will affect the microbiota. Hence in a unanimous controlled setting this study could not be done and also blinding cannot be done.

8. Acknowledgement

This study is a short communication extracted from a project wherein the financial support as grant-in-aid was provided by The Ministry of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homeopathy, Government of India(grant no. Z28015/22/2018-HPC (EMR)-AYUSH-A)

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