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Epigenetic Modifications among Children with Autism in Response to Phoenix Dactylifera: An Intervention Study

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Abstract

This study explored the influence of the regular daily intake of 5 pieces of Ajwa dates fruit (*Phoenix dactylifera*) for four months on modifications of epigenetic factors related to autism. An interventional exploratory study was conducted on 31 autism-diagnosed children aged 3-12 years. Methylation-specific PCR was used to determine the methylation density of Olfactory receptor family 2 subfamily L member 13 (*OR2L13*), Reelin (*RELN*), Proline-Rich Transmembrane Protein 1 (*PRRT1*), and index of global methylation (Long interspersed nucleotide element-1, *LINE-1*). Quantitative real-time PCR was used to test the change in the expression of DNA methyltransferase 1 (*DNMT1*) and chromodomain helicase DNA-binding protein 8 (*CHD8*), the expression of plasma *miR-146a* and *miR-146b*. The study also investigated the clinical, microbial, and biochemical factors' responses to Phoenix dactylifera intake. In response to Ajwa dates fruit intake, a significant decrease in the expression of *DNMT1*, was shown in 64.5% of children (P=0.012). *LINE-1*, *OR2L13*, and *PRRT1* showed significant changes in their methylation density. A significant decrease was observed in the pattern of *RELN* methylation status and the mean expression fold change of *MiR-146a* for 74.2% and 38.7% of children respectively. *Phoenix dactylifera's* regular intake plays a role in epigenetic modification among children with autism.

Keywords: Ajwa dates fruit; Autism; epigenetics modulation; DNA methylation, Expression of *DNMT1* and *CHD8*; Expression modulation of plasma *miR-146a* and *miR-146b*; *Phoenix dactylifera*

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Introduction

Autism spectrum disorder (ASD) is a terminology describing a wide range of abnormal behaviors and psychological difficulties in communication and social cooperation (1). Globally, ASD affects around 1in 68 children (2). A recent Egyptian study found that the overall prevalence of children aged 1 to 12 years who are at high risk of ASD is 3.3% [3].

Neuropathological studies suggest that individuals with Autism spectrum disorder (ASD) have alterations in neuronal organization, and neurotransmitter pathways [4, 5]. The odds of having obesity children with ASD carried more odds for having obesity compared to their healthy developing peers [6]. Accordingly, it was postulated that genetic, nutritional, and environmental factors play their role in ASD aetiology, either directly or via epigenetic modifications by influencing fetal or early postnatal brain development. Meanwhile, genetic and environmental factors and their interaction can influence also epigenetic change [7, 8]. Environmental factors can affect epigenetic marks prenatally and throughout life, including smoking, stress, and nutrition [7, 8]. Many pieces of evidence have suggested the involvement of epigenetics in ASD pathogenesis. For example, genetic screening revealed that the genes that have epigenetic functions constitute a sizable proportion of ASD candidate genes [7]. Other evidence is that some ASD-associated chromosomal abnormalities were identified in imprinted regions.

Epigenetic mechanisms can regulate the pattern of gene expression without changing the primary DNA sequence. Epigenetics has the ability to modulate and regulate the expression of genes through various epigenomic marks, such as DNA methylation and histone modifications which represent key mechanisms of epigenetics. These marks change the chromatin conformation by making it more compact and inhibit the ability of transcription factors to bind to the DNA, or opening it to allow transcription factor binding [7]. Non-coding RNA, particularly miRNA, is considered an epigenetic modulator due to its role in regulating target genes without changing the sequence. It was found that there is a miRNAs-epigenetic feedback loop, where miRNAs can regulate genes coding for enzymes involved in epigenetic machinery such as DNA methyl transferases (*DNMTs*) [9], and miRNAs can be regulated by epigenetic mechanisms such as DNA methylation, and histone modifications [10]

Methylation analysis of post-mortem brain samples of ASD individuals showed differentially methylated loci, these loci differ between various brain regions. These loci were hypomethylated at the promoter region, and hypermethylated at the gene body and intergenic sequence, however, identification of global methylation difference between brains of ASD and controls is limited [11].

Nutrition is recognized as one of the factors that have the ability to modify epigenetic marks. Ajwa dates fruit (*Phoenix dactylifera*) is known as a rich source of minerals and polyphenols [12, 13], and these compounds are verified to play a role in epigenetic changes. Minerals are proven to have an obvious epigenetic effect through epigenetically active enzymes that require minerals as cofactors [14]. Polyphenols can regulate gene expression in the hippocampus by epigenetic mechanisms and can be used in the management of many neurological diseases [15].

Hussein and his colleagues reported in their recent experimental study that palm date fruit extract could be a potential candidate for reducing Valproic Acid (VPA)-induced autistic-like behavioral changes in rats. They proposed the potential underlying mechanisms might involve reduction of the brain inflammation, oxidative stress, apoptosis, and upregulation of the antioxidant genes Nrf2 and HO-1, Sirt-1, and autophagy in the cerebellum and hippocampus [16].

This study aimed to test the role of Ajwa palm date fruit intake in Epigenetics modification among children with autism who were subjected to continuous daily consumption of five dates fruit for four months. This aim was achieved through the following analyses: The methylation percentage of Olfactory receptor family 2 subfamily L member 13 (*OR2L13*), Reelin (*RELN*), Proline-Rich Transmembrane Protein 1 (*PRRT1*), and index of global methylation (*LINE-1*), the expression profile of DNA methyltransferase 1 (*DNMT1*) and chromodomain helicase DNA-binding protein 8 (*CHD8*). Expression of plasma miR-146a and miR-146b was also tested. In addition, this study aimed to investigate the influence of the clinical, microbial, and biochemical characteristics on epigenetics modification's responses to Ajwa palm date fruit intake among participants.

Subjects and Methods

Study type: Intervention clinical exploratory study (pilot study).

Study Duration: The whole study has been conducted over one year between June 2021 and June 2022.

Study Setting:

Participants who were enrolled in the study were among children with autism attending both the outpatient clinics of Children with Special Needs outpatient clinic and Clinical Genetics Department clinic, Medical Research Centre of Excellence, National Research Centre (NRC) of Dokki – Giza governorate, Egypt.

Target Group and Inclusion Criteria

A cohort group of 31 children with autism on Ajwa dates fruit for four months was followed up. The clinical diagnosis of children with autism was based on the criteria for the autistic disorder as described in the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition, [17] and the Childhood Autism Rating Scale (CARS) [18]. Children with confirmed diagnosis of ASD were enrolled in the study according to inclusion and exclusion criteria. Specified paediatricians confirmed the enrolment criteria by complete history taking added to thorough clinical assessment. Inclusion criteria: 1) children aged three to twelve years of age at the beginning of the trial; 2) approved to be not allergic to dates fruit (confirmed by one piece of dates trial before start); 3) Accepts the daily intake of dates fruit for 4 months; 4) Not joining any other study 5) No main changes in medical treatments in the preceding 2 months, and not planning for any major changes throughout the study. Exclusion criteria: 1) All subjects with major medical problems or comorbid syndromes; 2) Patients who consumed probiotics and/or dates fruit for at least four weeks before the current study and/or antibiotics or laxatives in the last six months before the study; (3) History of seizure disorder or gross neurological deficit; 4) comorbid diagnosis: Fragile X syndrome, tuberous sclerosis, phenylketonuria. The formerly mentioned conditions were excluded from the study after history taking and checking positive reports from the clinical genetic team from NRC. The clinical genetic team was authorized with any suspected genetic disease associated with any physical features. The study approach and its phases are summarized in **Figures 1 and 2**.

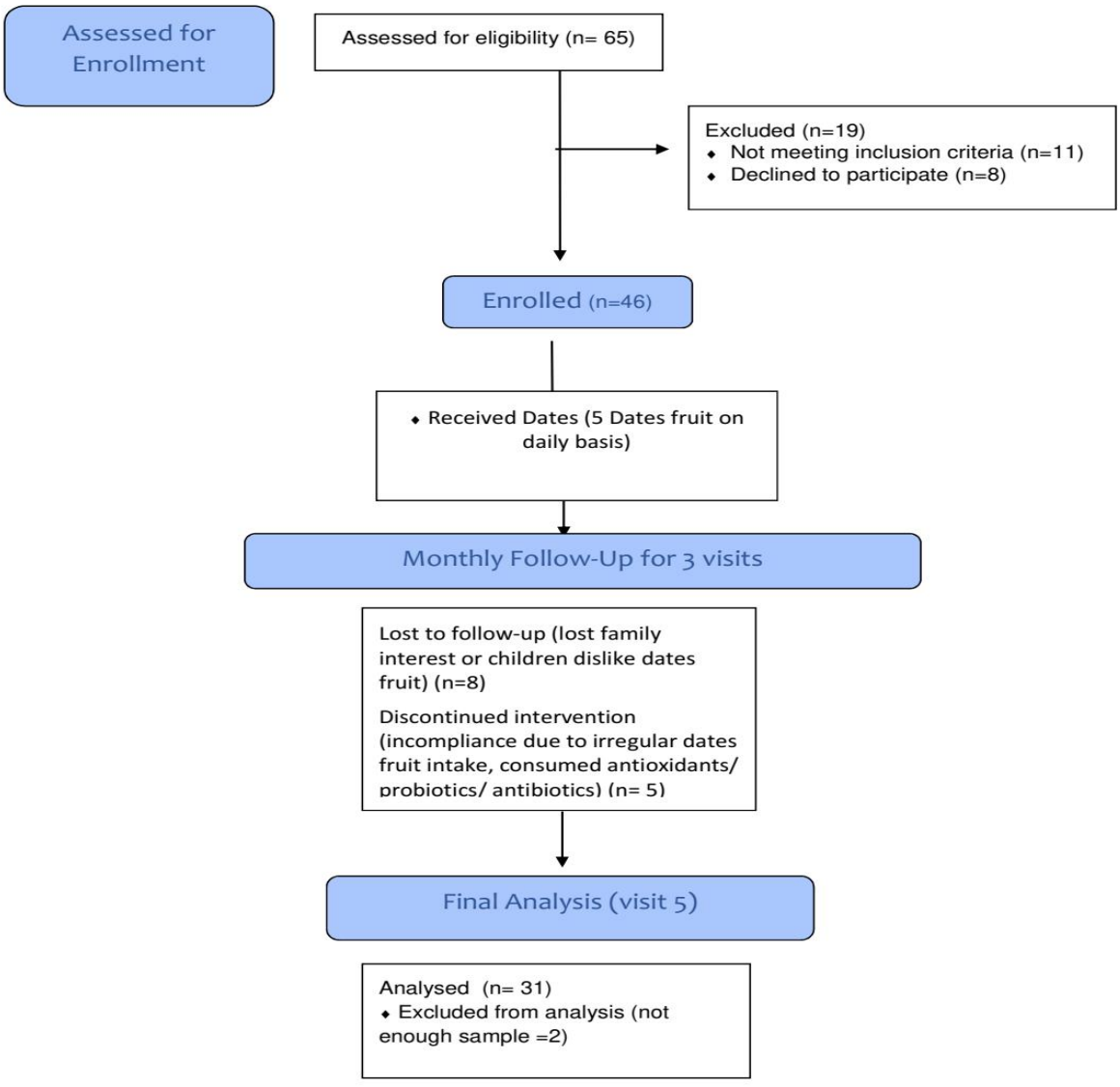

Figure 1: Flow Diagram for the study approach

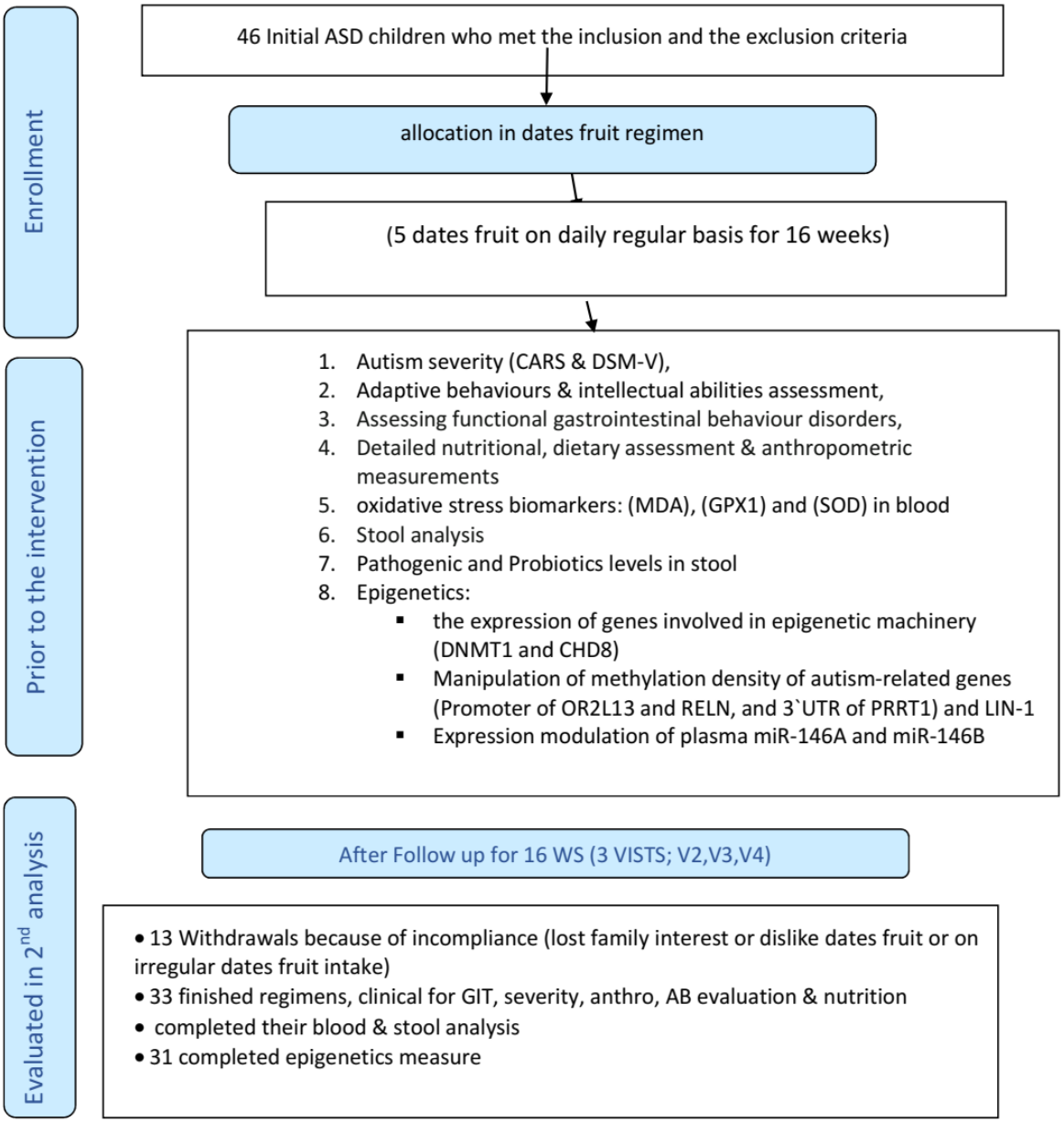

Figure 2: Flow Diagram for the time line and the flow of the study

Sample size: Thirty-two children with autism was calculated to achieve an eighty-five % power to detect a change prior to and after the study based on the assumption that improvement is expected to be one and a half times or more with the intake of dates fruit. The test statistic used is the two-sided Z test with pooled variance. The error of the test was targeted at 0.05. The significance level truly attained by this design was 0.0587 [19-20].

Intervention

The intervention included: Ajwa dates fruit intake. All participants were requested not to change their regular habitual diet for the duration of the study.

Children enrolled received Ajwa palm date fruit daily for 16 weeks as five pieces of date fruit/ day (each about 10 -15 gm). Ajwa palm date fruit was taken with breakfast or between breakfast and lunch as a daily dose (without drinking any tea after it for at least one hour).

However, the current proposed trial is novel and designed to assess the impact of date fruit on autism. The determined extent for this trial is chosen to allow sufficient time for dates fruit to cause an effect while ensuring no gain in weight.

The chosen number of pieces of dates for intake was based on two rationales: 1st: a previous study was done by Al Jaouni and his colleagues in 2019 and proved the effect of three pieces of Ajwa on improving outcomes among 26 children hospitalized with cancer [21]. 2nd : the recommendations by Prophet Mohammed (Peace Be upon Him) [22] he said that "dates cure several disorders, Saud (R.A) narrated that I heard Allah's Apostle saying, "If Somebody takes five to seven Ajwa dates in the morning, neither magic nor poison will hurt him that day" [23]. Prophet Mohamed recommended the intake of this dose irrespective to age. Accordingly, we went to the trial with five pieces of dates

Ajwa Dates fruit:

The Ajwa Dates fruit (Phoenix dactylifera) used during this clinical trial was Saudi Dates known as Al-Madinah Ajwa, these dates are organic Dates. Ajwa dates are famous for its very high nutritive and pharmacological values [12, 13]. 15 Ajwa date fruit (average 100 gm) were analysed before the study. The composition of 15 of the flesh Ajwa date fruit was carried out at the Safe Food Unit in the National Research Centre of Egypt. The authors did this analysis prior to the study to provide solid base of data and sufficient evidence to justify the testing of contents of the serving units (5 dates) and the required daily allowance/intake of these elements in children aged 3-12 years were shown in the table. It shows that Ajwa date flesh is a high-energy food due to its rich sugar contents that reached 74.2% of dry weight. Glucose and fructose (the reducing sugars) are the major sugars, while sucrose (non-reducing sugar) represents a minor percentage in composition. Ajwa dates contained low concentrations of protein and lipid (2.92% and 1.2%, respectively). However, the amino acids analysis (Table 1) of Ajwa flesh showed a high percentage of essential and non-essential amino acids. The major essential amino acids distinguished were lysine (72 mg/100 g), valine (65mg/100g), and leucine (58 mg/100 g), in addition to threonine, isoleucine, phenylalanine, and low concentrations of methionine, and histidine. Higher concentrations of aspartic acid, and glutamic acid are present, with the rest of the non-essential amino acids (Table 1). Amino acids play central roles as building blocks of proteins, synthesis of neurotransmitters, and as intermediates in metabolism [37].

Table 1 shows also that the Ajwa date contained significant amounts of minerals. The potassium Dates' fruit in children with autism. The 15 samples of Ajwa dates were free of pathogenic bacteria. The sample preparation and analysis were done according to the Association of Official Analytical Chemists (AOAC) [24] and to Nowatzk et al [25] to minimize sample-to-sample variations within and between laboratories. Analysis of the composition of the flesh of Ajwa dates fruit was done according to the following standard methods: Total fiber contents were determined according to Garcia et al [26], and Ash content was determined according to fennema [27]. Vitamin A was carried out according to Hiroyuki Moriyama et al [28]. Ascorbic acid (Vitamin C) was determined according to Evered [29]. Vitamin B1, B2, and B3 were determined according to Radaamidzic and his colleagues [30]. Minerals were measured according to Musa Özcan [31]. All analyses of the total amount of carbohydrate were determined using the phenol–sulphuric acid method in duplicate [32] Sugar concentration was calculated based on peak area measurements [33]. Amino Acid Analysis was determined using a Beckman 6300 system [34]. The total flavonoid content of the date extracts was measured according to the colorimetric assay of Kim, Jeong, and Lee (2003) [35] and the total phenolics were determined as described by Al-Farsi et al [36].

Table 1 shows the chemical composition of date flesh per 100 grams of dry weight, according to the analysis done at the Safe Food Unit in the National Research Centre (NRC) of Egypt. The nutritive concentration was the highest (476.5 mg/100 g), followed by calcium (187.4 mg/100 g), magnesium (150.5 mg/100 g), phosphorus (27.1 mg/100 g), and sodium (7.5 mg/100 g). There were low concentrations of iron, selenium, and zinc. Following other studies, we found that Ajwa date fruit is the richest source of dietary minerals among all other date varieties [38, 39]. Vitamins, such as vitamin A, thiamine (Vit B1), niacin (Vit B3), pantothenic acid (Vit B5), vitamin B6, riboflavin (Vit B2), ascorbic acid (Vit C), and Folic acid were detected in our Ajwa dates samples in percentages similar to what has been reported in previous studies [40, 41]. These minerals and vitamins are essential for cell functions and biochemical reactions [13].

The major phenolic compounds and types in Ajwa were also analyzed at NRC (Table 1). These phenolic compounds are proven to be potent antioxidants with strong bioactivities against several bacterial pathogens [42, 43]. The phenolic compounds commonly detected in Ajwa date fruits have all been stated to provoke strong antioxidant activities with its potential to improve oxidative stress–related diseases and infectious diseases as previously reported [44].

Table 1: Composition (g/100 g dry weight) of the flesh of Ajwa date fruit according to the analysis done at the Safe Food Unit in the National Research Centre of Egypt

Composition	/100 g dry weight	/Serving (5 dates/day)	DRI Children <6 years*	DRI Children 6-12 years*
Chemical composition (gm)				
Moisture	22.9	10.3 – 17.2		
liquid	0.48	0.22 – 0.36		
Protein	2.92	1.31 – 2.19	24 g/day or (0.87g/kg/day)	24 g/day Or (0.76g/kg/day)
Fat	1.2	0.5 – 0.9	39 g/day 30-40% of Total energy intake	62 g/ day 25-35 % of Total energy intake
Total Dietary fibers (TDF)	7.8	3.5 – 5.9	25 g/day	28 g/day
Ash	3.44	1.55 – 2.58		
Total sugar (Carbohydrate) (gm)	74.2	33.4 – 55.7	200 g/day	225 g/day
Glucose	50.1	22.6 – 37.6		
Fructose	47.3	21.2 – 35.5		
Sucrose	2.6	1.2- 1.95		
Minerals (mg)				
Calcium	187.4	84.3 – 140.6	800 mg/day	1200 mg/day
Phosphorus	27.1	12.2 – 20.3	460 mg/day	500 mg/day
Potassium	476.5	214.4 – 357.4	3 mg/day	3.8 mg/day
Sodium	7.5	3.4 – 5.6	1.0 g /day	1.2 g /day
Magnesium	150.5	67.7 – 112.9	80 mg/day	130 mg/day
Selenium	0.36	0.16 – 0.27	20 µg /day	30 µg/ day
Zinc	0.35	0.16 – 0.26	10 mg/day	10 mg/day
Iron	1.23	0.55 – 0.92	10 mg/day	10 mg/day
Vitamins (mg)				
Vit A	0.4	0.18 – 0.3	500µg /day	700 µg /day
Thiamine (Vit B1)	0.068 mg	0.031 – 0.051 mg	0.6 mg/day	0.9 mg/day
Niacin (Vit B3)	1.369 mg	0.616 – 1.027 mg	8 mg/day	12 mg/day
Pantothenic acid (Vit B5)	0.729 mg	0.328 – 0.547 mg	2 mg/day	3 mg/day
Vitamin B6	0.198	0.089 – 0.149	0.6 mg/day	1 mg/day
Riboflavin (Vit B2)	0.076	0.034 – 0.057	0.6 mg/day	0.9 mg/day
Ascorbic acid (Vit C)	10.2	4.59 – 7.65	45 mg/day	45 mg/day
Folic acid (folacin)	0.008	0.004 – 0.006	200 µg /day	300 µg /day
Amino Acid (mg/kg per day)**				
Essential Amino Acid (mg)**				
Threonine (Thr)	52	23.4 - 39	20 mg/kg per day	
Valine (Val)	65	29.3 – 48.8	24 mg/kg per day	
Methionine (Met)	26	11.7 – 19.5	19 mg/kg per day	
Isoleucine (Iso)	45	20.3 – 33.8	19 mg/kg per day	
Leucine (Leu)	58	26.1 – 43.5	42 mg/kg per day	
Phenylalanine (Phe)	46	20.7 – 34.5	33 mg/kg per day	
Histidine (His)	25	11.3 – 18.8	14 mg/kg per day	
Lysine (Lys)	72	32.4 - 54	38 mg/kg per day	
Tryptophane §	44			
Other non-essential Amino Acid (mg)**				
Aspartic acid (Asp)	185	83.3 – 138.8		
Cysteine (Cys)	44	19.8 - 33		
Serine (Ser)	60	27 - 45		
Glutamic acid (Glu)	210	94.5 – 157.5		
Proline (Pro)	87	39.2 – 62.3		
Glycine (Gly)	83	37.4 – 62.3		
Alanine (Ala)	83	37.4 – 62.3		
Tyrosine (Tys)	45	20.3 – 33.8		
Arginine (Arg)	83	37.4 – 62.3		
Phenolic acid composition (mg)				
Total phenols	482.2	216.99 – 361.7		
Caffeic acid	0.051	0.023 – 0.038		
Ferullic acid	3.00	1.35 – 2.25		
Protocatechuic acid	2.10	0.95 – 1.58		
Catechin	0.71	0.32 – 0.53		
Gallic acid	14.0	6.3 – 10.5		
p-coumanic acid	3.41	1.53 – 2.56		
Chlorogenic acid	0.19	0.09 – 0.14		
Resorcinol acid	0.04	0.02 – 0.03		
Flavonoids glycosides				
Total Flavonoids	2.84	1.28 – 2.13		
Quercetin	1.19	0.54 – 0.89		
Luteolin	0.05	0.02 – 0.04		
Apigenin	0.25	0.11 – 0.19		
Rutin	0.88	0.40 – 0.66		
Iso-quercetin	0.40	0.18 – 0.30		

* The average daily dietary intake of Ajwa dates fruit was analyzed for calculating the nutrients intake adequacy and their percent out of the Daily Recommended Intake (DRI**)** [45, 46].

**Estimated amino acid requirements for daily intake nutrient (mg/kg per day)

§ This amino acid is added according to previous research [38]

Study Visits, safety, and adverse events

Participants underwent 5 visits. The first one (Visit 1) included an initial evaluation of the inclusion, exclusion criteria and parental signing of the informed consent process. A thorough medical history, current drug intake, any supplement, and meticulous demographic data were collected. Paediatrician forms were also delivered to the parents to confirm the inclusion/exclusion criteria. Throughout the first visit, baseline, dietary assessment & anthropometric measurements, Stool samples were collected. Additionally, Real-time PCR was performed to evaluate the autistic children' gut microbiota. The existence of probiotics in autistic children was studied to assess the alterations in their levels due to the trial. A blood sample was collected to determine the concentration of three serum markers: Malondialdehyde (MDA), glutathione peroxidase (GPX1), and superoxide dismutase (SOD). The following also was done: Methylation analysis for the *LINE-1*, promoter of *OR2L13* and *RELN*, and *3 UTR* of *PRRT1*, Quantitative real-time PCR for *DNMT1* and *CHD8* expression analysis, and Quantitative real-time PCR (Polymerase Chain Reaction) for *miR-146a* and *miR-146b* expression analysis for all children with autism who were enrolled in the study.

2nd, 3rd, and 4th visits (follow-up visits) were done after the first visit by one, two, and three months respectively. The principal aim of these visits was to evaluate patients' compliance, document hazardous or unexpected events, and to confirm continuous Dates' fruit intake.

5th visit was the last one that took place 16 weeks after the initial baseline visit. it was the evaluation visit. similar tests were accomplished in similar sequence as stated earlier in the first visit. Stool and blood samples were obtained during the visit to assess the effect of consuming the Dates' fruit for four months on the microbiota, oxidative stress markers, the expression of genes involved in epigenetic machinery (*DNMT1* and *CHD8*), manipulation of methylation density of autism-related genes (Promoter of *OR2L13* and *RELN*, and *3`UTR* of *PRRT1)* and *LIN-1* and the expression modulation of plasma *miR-146a* and *miR-146b* (Fig 1 and 2).

Assessment and evaluation tools

The assessment was done prior to the study intervention, whereas the evaluation was done four months after the intervention.

Assessment of the baseline sociodemographic characteristics was done through detailed history taking. Neurological assessment was done through thorough clinical examination.

Assessing symptoms of functional gastrointestinal behavior disorders (FGID) included: constipation, diarrhea, gaseousness, bloating, gastroesophageal reflux, abdominal pain, and vomiting [47].

Thorough **dietetic history and behaviour** were performed using Diet Quality index assessment questionnaire [48] to assess the risk of malnutrition.

The anthropometric measurements including body weight (kg) and height (cm), all were determined prior to the beginning of the study using a digital scale and a wall height measuring device while the child wearing minimal clothing with bare feet. Body mass index (BMI, kg/m^2) was calculated as body weight (in Kg) divided by body height (in m^2), then, all the anthropometric measures were converted to z-scores specific age and gender of the child (standard deviation units, SDU) based on the World Health Organization (WHO) child growth standards [49]. BMI z-scores of autistic children were compared pre and post the trial to assess weight gain.

Assessment of Adaptive Behaviour: In all phases of the study, the adaptive behavior was evaluated using the Adaptive Behaviour Assessment System, Third Edition (ABAS-3). The ABAS-3 covers 3 broad domains using ten skill areas inside these domains. The skills focus on daily essential activities, meeting environmental demands, self-care, and interaction with others efficiently. The adaptive domains are Conceptual (CON), Social (SOC), and Practical (PR). The motor skills were restricted to children 5 years old and younger. Collectively, the 3 domains scores form the General Adaptive Composite (GAC) and afford the overall estimation of adaptive behaviour. The average score of GAC or any of the three main domains ranged from 90 to 109 [50].

Reliability of the ABAS-3 was studied from numerous perspectives, with the adaptive scaled scores, adaptive standard scores, and GAC scores performing well on indexes of internal consistency, standard error of measurement, test–retest reliability, interrater reliability, cross-form consistency, and alternative-forms reliability [51]. The scaled and composite scores had a correlation with scores from the ABAS-2 and Vineland adaptive behavior scale-II, which provides evidence of convergent validity. Additionally, ABAS-3 scores differentiate individuals with normal development from those with autism [52].

Biochemical Tests

Blood and Faecal samples were collected before the start of the interventions and after (16 weeks) of completing the intervention for evaluating the stool microbiota and the serum antioxidant markers levels. In vitro, quantitative determination of three markers;

Malondialdehyde (MDA), glutathione peroxidase (GPX1), and superoxide dismutase (SOD) concentrations in serum was performed using Enzyme-linked Immune Sorbent Assay (ELISA) according to [53, 54, 55]. The quantity of the pathogenic bacteria was detected in the faecal samples of all enrolled children using the real-time PCR. The following pathogenic bacteria were assessed in stool: Klebsiella, Proteus, Shigella, Enterobacter, and Salmonella. Recognition of beneficial microbiota (probiotics: Lactobacillus and Bifidobacteria strains) was performed using a DNA extraction Kit [56, 57].

Methylation analysis

DNA was extracted using standard salting-out protocol from blood samples stored at -80 oC. The extracted DNA was exposed to bisulfite modification using the EpiTect Bisulfite kit (Qiagen) according to manual instructions. The Initial starter quantity of DNA was 1.3 µg and the final elution volume was 40 µl. The converted DNA was used for methylation-specific PCR (MSP), in which 2 different sets of primers (methylated and unmethylated) were used and amplified separately for each gene. Each set of MSP reactions was carried out in a 15 µl reaction using 7.5 µl of maxima SYBR green master mix (Thermo Scientific), 2 µl of treated DNA, and 150 nM of every primer. The percentage of methylation was calculated using the CT method according to the following equation: The percentage of methylation was calculated using the CT method according to the following equation: the percentage of methylation percentage = 100/[1+2ΔCt(methylated-unmethylated)] %" to be The percentage of methylation was calculated from the change of CT values according to the following equation: methylation percentage (%) = 100/[1+2ΔCt(methylated-unmethylated)] where ΔCt was calculated as follow ΔCt = (methylated - unmethylated) [58, 59]. Primers for *LINE-1*, promoter of *OR2L13* and *RELN*, and *3 UTR* of *PRRT1* were designed by the free online methprimer program (http://www.urogene.org/methprimer) using CpG island prediction for primer selection. (sequence of used primers is shown in **Table 2**). A duplicate calibrator sample was used in all runs to allow for a comparison of the results across all runs.

Table 2: List of primers used for real-time PCR and MSP

Gene	sequence 5→3
CHD8 F	CTCAGAATCAGAGTTTCTCAAGGAC
CHD8 R	CCAAAATAGCCCGATAGTATTTCTT
DNMT1 F	GAGAACACCCACAAGTCCACTC
LINE-1 M-F	GAGGTATTGTTTTATTTGGGAAGC
LINE-1 M-R	TACTAACAATCAACGAAATTCCGTA
LINE-1 U-F	AGGTATTGTTTTATTTGGGAAGTGT
LINE-1 U-R	TACTAACAATCAACAAAATTCCATA
DNMT1 R	CGAGGAAGTAGAAGCGGTTG
OR2L13 MF	GTTTAAAGGTGATATGTATGGATCG
OR2L13 MR	TACTTAAACCCTACCAAAAAAACGT
OR2L13 UF	GGAGTTTAAAGGTGATATGTATGGATT
OR2L13 UR	ACTTAAACCCTACCAAAAAAACATC
PRRT1 MF	GGGGTCGTTAGATTTGGATATATC
PRRT1 MR	AAACCAAACCGAAATTACTCGA
PRRT1 UF	GGGTTGTTAGATTTGGATATATTGT
PRRT1 UR	AAAAACCAAACCAAAATTACTCAAT
RELN MF	TCGAGTTTGTTAATTTTATTTTCGT
RELN MR	GCCGATTTCTTTATCTAAACCG
RELN UF	TGAGTTTGTTAATTTTATTTTTGT
RELN UR	CACCAATTTCTTTATCTAAACCAAC
miR-164a-5p	GTGTTTTTTTGTGAGAACTGAATTCCA
miR-146b-5p	TGTTTTTTTTTGTGAGAACTGAATTCCAT

Quantitative real-time PCR for DNMT1 and CHD8 expression analysis

Total RNA (including miRNA) was extracted from plasma using Direct-zol™ RNA MiniPrep (Zymo Research) per the manufacturer's instructions. The extracted RNA was reverse transcribed into single-stranded complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). For mRNA expression analysis, mixtures were prepared using the maxima SYBR green qPCR master mix (Thermo Fisher Scientific) according to manufacturer recommendations.

GAPDH was used as an internal reference gene to normalize gene expression. Primer sequences of DNMT1 and CHD (listed in Table 2) were designed with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). Results were expressed as a ratio of reference to target gene using the $2^{-\Delta Ct}$ method.

Quantitative real-time PCR for miR-146a and miR-146b expression analysis

Reverse transcription into cDNA was carried out using miScript II RT Kit (Qiagen). For miRNA expression analysis, mixtures were prepared by miScript SYBR Green PCR master mix according to the manufacturer using included universal (reverse) primer, while forward primers of *miR-146a* and *miR-146b* were designed by the online miRNA primer design tool (http://genomics.dote.hu:8080/mirnadesigntool/). Expression was normalized to RNU6B. Results were expressed as a ratio of reference to target gene using the 2-ΔCT method. Primers were listed in Table 2

Statistical Analysis:

After data entry of all completed questionnaires, Statistical analysis was completed using the Statistical Package of Social Software program (SPSS), version 20. Descriptive statistics were used for quantitative data, as mean and standard deviation for normally distributed data while median and IQR (interquartile range, 1st quartile-3rd quartile) for abnormally distributed data. Though, numbers and percentages were used to describe qualitative values. Pre and post the intervention, related indices were compared for the studied group. Tests of significance were used such as: Pearson's Chi-square test ($\chi 2$) and Z test (for qualitative data), paired t-test and Wilcoxon Signed Ranks Test (for continuous data between the before and after intervention), while t-test and Mann-Whitney test (for continuous data between means and medians of 2 groups individually). P-value < 0.05 was considered statistically significant, while P-value < 0.01 was considered highly statistically significant.

Results

Sociodemographic, clinical and laboratory characteristics of the included children with autism aged 3 – 12 years. **Table 3** shows the characteristics of the enrolled children with autism. A total of 31 children completed the four-month intake of Ajwa palm date fruit. More than half of children aged six years and above (58.1 %). The boys were significantly higher than girls (77.4 % versus 22.6 % respectively). The mean age of mothers at birth was 28.3 ± 4.7 while fathers were 32.4 ± 4.9. Regarding education levels, 54.8 % of fathers and mothers had completed their university degrees. Most of the fathers were engaged in non-risky administrative employment (83.9%).

Table 3: Sociodemographic characteristic of the enrolled children with autism

Characteristics	Children with autism n=31	P value
Age of the children (years) (mean ± SD)	6.9 ± 2.7	
Age of the children (no , %)		
< 6 years	13 (41.9)	0.204
>= 6 years	18 (58.1)	
Gender (no (%)		
Boys, no (%)	24 (77.4)	**<0.001****
Girls, no (%)	7 (22.6)	
Age of mother at time of birth (mean ± SD)	28.3 ± 4.7	
Age of father at time of birth (mean ± SD)	32.4 ± 4.9	
Mother Education (no (%)		
Illiterate/ Read and write/ primary/ Prep	5 (16.1)	**0.001****
High School and technical	9 (29.1)	**0.039***
University or higher	17 (54.8)®	
Father Education (no (%)		
Illiterate/ Read and write/ primary/ Prep	6 (19.4)	**0.004****
High School and technical	8 (25.8)	**0.020***
University or higher	17 (54.8)®	
Father employment (no (%)		
Risky employment	5 (16.1)	**<0.001****
Non-risky-Administrative	26 (83.9)	

Risky employment: 1-plumber, 2-welder, 3-painter, 4- builder, 5-manufacture of decorating or table tools or 6-repair of electrical appliances, #Median (1st quartile - 3rd quartile)
®Reference
Test of significance is: z test between proportions
*Significant < 0.05, **Highly significant < 0.01

The following clinical and laboratory characteristics of the studied subjects were evaluated before and after the intake of dates fruit and presented in **Table 4**. Clinical characteristics included: Autism severity symptoms as assessed by CARS level and DSM-V levels, adaptive behavior symptoms, the risk of malnutrition, the growth indices of the anthropometric characteristics including Weight/age z-score, Weight/height z-score, BMI/age z-score, GIT risk index and the existence of pathogenic organisms in enrolled children' stools.

In response to Ajwa palm date fruit intake for 4 months, a significant reduction has been detected concerning the mean level of CARS level (36.1 ± 3.2 before versus 33.4 ± 3.2 after the intervention). Concerning the severity scale of DSM-V, a highly significant decrease in the number of those who suffer from severe autism (35.5 % before versus 12.9 % after the intervention).

When assessing the pathogenic bacteria which present in the stool of children with autism, there was also a significant reduction in the presence of the pathogenic organism due to intake of dates fruit for four months.

The faecal samples from enrolled children were tested to compare beneficial bacteria's level; Bifidobacteria and Lactobacillus species prior to and after the intervention and presented in table 4. The stool PCR of children with autism showed significantly increased levels of the mean colony counts of Lactobacilli due to the intervention.

A significant decrease in the proportion of oxidant to antioxidant serum blood levels has been observed in response to Ajwa palm date fruit intake for four months. The improvement was assessed by measuring both the median balances' ratios of MDA/SOD (41.7 before versus 23.7 after the intervention) and MDA/GPX (136.4 before versus 56.7 after the intervention).

Table 4: Clinical and laboratory characteristic of the enrolled children with autism pre- and post-intervention with Ajwa palm date

Characteristics	Baseline n=31	After Intervention n=31	P value
Mean CARS level (mean ± SD)	36.1 ± 3.2	33.4 ± 3.2	**<0.001****
DSM-V Severity Severe Mild and Moderate	 11 (35.5) 20 (64.5)	 4 (12.9) 27 (87.1)	**0.038***
Baseline means of adaptive behaviour (mean ± SD)			
Conceptual domain	53.5 ± 6.4	58.3 ± 14.4	0.078
Social domain	55.4 ± 6.4	59.9 ± 12.8	0.056
Practical	59.1 ± 11.7	65.4 ± 17.5	0.103
General adaptive composite score	54.3 ± 10.7	59.5 ± 15.5	0.061
Risk of malnutrition (no (%) At risk At potential risk	 10 (32.3) 21 (67.7)	 6 (18.5) 25 (81.5)	0.246
Weight/age z-score (mean± SD)	1.0 ± 1.4	1.2 ± 1.5	0.580
Weight/height z-score (mean± SD)	1.2 ± 1.3	1.3 ± 1.8	0.790
BMI/age z-score (mean± SD)	1.6 ± 1.4	1.6 ± 0.5	0.942
Obese children (no (%) Obese Non obese	 10 (32.3) 21 (67.7)	 13 (41.9) 18 (58.1)	0.430
Presence of Pathogenic Organisms (+ve) (no (%) Participants with +ve results Participants with -ve results	 21 (67.7) 10 (32.3)	 13 (41.9) 18 (58.1)	**0.041***
Log level of lactobacillus Spp.[$] (no (%) Deficiency Normal	 17 (54.8) 14 (45.2)	 9 (29.0) 22 (71.0)	**0.040***
Log level of Bifidobacterium Spp.[$] (no (%) Deficiency Normal	 17 (54.8) 14 (45.2)	 13 (41.9) 18 (58.1)	0.309
Median Balance MDA/SOD (Median, IQR)[#]	41.7 (25.0-68.7)	23.7 (13.4-48.2)	**0.001****
Median Balance MDA/GPX (Median, IQR)[#]	136.4 (78.6-233.0)	56.7 (27.2-100.0)	**<0.001****

[$] Probiotics: Lactobacillus spp. log ≤6.00 = deficiency, Bifedobacetrium spp. log ≤5.93 = deficiency

[#]Median (1st quartile - 3rd quartile)

Test of significance are: paired t test between pre and post means of the same group, X^2 test between groups, Wilcoxon Signed Ranks Test between pre and post medians of the same group

*= p-value significant at <0.05, **=p-value highly significant at <0.01

Ajwa palm date fruit intake affects the expression of genes involved in the epigenetic machinery

Table 5 shows the expression of *DNMT1* and *CHD8* in plasma samples of studied children with autism before and after the regimen. As a result of the interventions, 30 subjects exhibited a significant change in the expression of *DNMT1*, a significant increase was shown in 10 cases (*P*=0.019) and a significant decrease was observed in 20 cases (*P*=0.012). Regarding *CHD8*, a slight increase was displayed in 9 cases (*P*=0.082) and a borderline

significant decrease was shown in 12 cases (*P*=0.052).

Manipulation of methylation density of autism-related genes and *LINE-1* in response to Ajwa palm date fruit intake

Table 6 shows the mean methylation percentage of *LINE-1*, Promoter of *OR2L13* and *RELN*, and *3`UTR of PRRT1* for studied subjects before and after the regimen. *LINE-1*, *OR2L13*, and *PRRT1* showed significant changes in their methylation density for both increase and decrease patterns. While the increase of *RELN* methylation status was displayed only in 8 cases and was not significant; in contrast to the decrease pattern which was observed in 23 subjects and was highly significant to the level that made the significance extend to include the total number of patients.

Expression modulation of plasma *miR-146a* and *miR-146b* in response to Ajwa palm date fruit intake

The expression of *miR-146a* and *miR-146b* in the plasma of the candidate children with autism was analyzed before and after the regimen. A significant decrease was observed in the mean of expression fold change of *miR-146a* for 12 children in response to Ajwa palm date fruit intake **(*P*=0.04).** No significant change was found in the expression of *miR-146b* at both the increase and decrease patterns (table 7).

Table 5: Expression of *DNMT1* and *CHD8* in plasma samples of studied children with autism before and after Ajwa palm date fruit intake

Gene	Mean of expression fold change		
	Before Mean ± SD	**After Mean ± SD**	***P* value**
DNMT1			
Total (n=31)	0.16 ± 0.21	0.09 ± 0.08	0.083
Increased (n=10)	0.06 ± 0.03	0.13 ± 0.08	**0.019***
Decreased (n=20)	0.22 ± 0.24	0.08 ± 0.07	**0.012***
CHD8			
Total (n=25)	0.11 ± 0.23	0.05 ± 0.05	0.181
Increases (n=9)	0.00 ± 0.04	0.07 ± 0.07	0.082
Decreased (n=12)	0.22 ± 0.29	0.03 ± 0.03	0.052

The expression fold was analyzed by quantitative real time PCR according to 2^-Δct method.
Test of significance between before and after: paired t test between two means
Sustained cases were not included in the calculation, *= p-value significant at <0.0

Table 6: Manipulation of methylation density of autism-related genes and *LINE-1* in response to Ajwa palm date fruit intake

Parameter	Mean of methylation percentage		
	Before Mean ± SD	**After Mean ± SD**	**P value**
LINE-1			
Total (n=31)	91.02 ± 4.68	91.80 ± 4.63	0.496
Increased (n=18)	89.25 ± 4.79	93.98 ± 2.46	**<0.001****
Decreased (n=13)	93.47 ± 3.34	88.79 ± 5.31	**0.003****
OR2L13			
Total (n=31)	47.81 ± 11.44	53.02 ± 12.04	0.056
Increased (n=21)	44.68 ± 11.09	57.62 ± 10.43	**<0.001****
Decreased (n=10)	54.38 ± 9.61	43.37 ± 9.40	**0.010***
RELN			
Total (n=31)	5.26 ± 5.55	2.18 ± 2.01	**0.004****
Increased (n=8)	1.22 ± 1.14	3.09 ± 2.93	0.068
Decreased (n=23)	6.67 ± 5.79	1.86 ± 1.54	**<0.001****
PRRT1			
Total (n=31)	18.99 ± 25.93	9.99 ± 8.92	0.082
Increased (n=11)	6.03 ± 4.60	15.24 ± 12.58	**0.038***
Decreased (n=20)	26.12 ± 29.99	7.10 ± 4.20	**0.008****

The expression fold was analyzed by quantitative real time PCR according to 2^-Δct method.
Test of significance between before and after: paired t test between two means
*= p-value significant at <0.05, **=p-value highly sig at <0.01

Table 7: Expression modulation of plasma miR-146a and miR-146b in response to Ajwa palm date fruit intake

Gene	Mean of expression fold change		
	Before Mean ± SD	After Mean ± SD	*P* value
MiR-146a			
Total (n=31)	3.76 ± 8.17	4.12 ± 11.18	0.711
Increased (n=16)	3.39 ± 9.41	5.86 ± 15.12	0.126
Decreased (n=12)	5.07 ± 7.50	2.69 ± 4.41	**0.040***
MiR-146b			
Total (n=29)	4.84 ± 12.27	4.97 ± 15.21	0.914
Increased (n=11)	5.79 ± 16.71	9.35 ± 23.85	0.157
Decreased (n=11)	6.76 ± 11.11	3.53 ± 6.38	0.062

Test of significance between before and after: paired t test between two means
Sustained cases were not included in the calculation, *= p-value significant at <0.05, **=p-value highly sig at <0.01

Table 8 shows the association between the improvement of some clinical and lab parameters and the change in the methylation pattern of some genes involved in epigenetic regulation as a result of Ajwa palm date fruit intake. The likelihood of having a decrease of the promotor of *OR2L13* was significant with the improvement of lactobacillus sup. The likelihood of having a decrease of *PRRT1* was significant with the improvement of GIT symptoms. Meanwhile, the likelihood of having an increase in *RELN* was significant with the improvement of the presence of microorganisms.

Table (8): Association between improvement of some clinical and lab parameters and change of methylation pattern of some genes involved in epigenetic regulation as a result of Ajwa date fruits intake

Parameters	Factors affecting *DNMT1*		Factors affecting *LINE-1*		Factors affecting *OR2L13*		Factors affecting *RELN*		Factors affecting *PRRT1*		Factors affecting *MiR-146a*	
	Increase n=10	Decrease n=20	Increase n=18	Decrease n=13	Increase n=21	Decrease n=10	Increase n=8	Decrease n=23	Increase n=11	Decrease n=20	Increase n=16	Decrease n=12
CARS												
• Not improved® (n=6)	1 (10.0)	5 (25.0)	3 (16.7)	3 (23.1)	4 (19.0)	2 (20.0)	3 (37.5)	3 (13.0)	2 (18.2)	4 (20.0)	4 (25.0)	2 (16.7)
• Improved (n=25)	9 (90.0)	15 (75.0)	15 (83.3)	10 (76.9)	17 (81.0)	8 (80.0)	5 (62.5)	20 (87.0)	9 (81.8)	16 (80.0)	12 (75.0)	10 (83.3)
P value	0.333		0.656		0.950		0.132		0.902		0.595	
DSM-V												
• Not improved® (n=4)	3 (30.0)	4 (20.0)	5 (27.8)	2 (15.4)	5 (23.8)	2 (20.0)	3 (37.5)	4 (17.4)	1 (9.1)	6 (30.0)	5 (31.3)	2 (16.7)
• Improved (n=27)	7 (70.0)	16 (80.0)	13 (72.2)	11 (84.6)	16 (76.2)	8 (80.0)	5 (62.5)	19 (82.6)	10 (90.9)	14 (70.0)	11 (68.7)	10 (83.3)
P value	0.542		0.415		0.813		0.241		0.183		0.378	
GIT severity symptoms												
• Not improved® (n=4)	1 (10.0)	2 (10.0)	2 (11.1)	2 (15.4)	2 (9.5)	2 (20.0)	0 (0.0)	4 (17.4)	4 (36.4)	0 (0.0)	3 (18.8)	0 (0.0)
• Improved (n=27)	9 (90.0)	18 (90.0)	16 (88.9)	11 (84.6)	19 (90.5)	8 (80.0)	8 (100.0)	19 (82.6)	7 (63.6)	20 (100.0)	13 (81.2)	12 (100.0)
P value	1.0		0.726		0.416		0.206		**0.004****		0.112	
Presence of any microorganisms												
• Not improved® (n=13)	4 (40.0)	9 (45.0)	8 (44.4)	5 (38.5)	7 (33.3)	6 (60.0)	0 (0.0)	13 (56.5)	4 (36.4)	9 (45.0)	8 (50.0)	4 (33.3)
• Improved (n=18)	6 (60.0)	11 (55.0)	10 (55.6)	8 (61.5)	14 (66.7)	4 (40.0)	8 (100.0)	10 (43.5)	7 (63.6)	11 (55.0)	8 (50.0)	8 (66.7)
P value	0.794		0.739		0.160		**0.005****		0.641		0.378	
Lactobacillus Supp. Log												
• Not improved® (n=9)	4 (40.0)	5 (25.0)	4 (22.2)	5 (38.5)	9 (42.9)	0 (0.0)	3 (37.5)	6 (26.1)	2 (18.2)	7 (35.0)	5 (31.3)	4 (33.3)
• Improved (n=22)	6 (60.0)	15 (75.0)	14 (77.8)	8 (61.5)	12 (57.1)	10 (100.0)	5 (62.5)	17 (73.9)	9 (81.8)	13 (65.0)	11 (68.7)	8 (66.7)
P value	0.398		0.326		**0.014***		0.540		0.324		0.907	
Improved MDA/SOD												
• Not improved® (n=13)	3 (30.0)	9 (45.0)	7 (38.9)	6 (46.2)	8 (38.1)	5 (50.0)	5 (62.5)	8 (34.8)	3 (27.3)	10 (50.0)	7 (43.8)	4 (33.3)
• Improved (n=18)	7 (70.0)	11 (55.0)	11 (61.1)	7 (53.8)	13 (61.9)	5 (50.0)	3 (37.5)	15 (65.2)	8 (72.7)	10 (50.0)	9 (56.2)	8 (66.7)
P value	0.429		0.686		0.530		0.171		0.220		0.576	
Improved level MDA/GPX												
• Not improved® (n=6)	2 (20.0)	4 (20.0)	3 (16.7)	3 (23.1)	4 (19.0)	2 (20.0)	2 (25.0)	4 (17.4)	1 (9.1)	5 (25.0)	3 (18.8)	3 (25.0)
• Improved (n=25)	8 (80.0)	16 (80.0)	15 (83.3)	10 (76.9)	17 (81.0)	8 (80.0)	6 (75.0)	19 (82.6)	10 (90.9)	15 (75.0)	13 (81.2)	9 (75.0)
P value	1.0		0.656		0.950		0.639		0.283		0.690	

®: Reference, Tests of significant was: X^2 test between groups, *Significant < 0.05, **highly significant < 0.01

Discussion

Considering that the bioactive contents of the food may provide protective epigenetic changes throughout life [8], we hypothesized a potential impact of Ajwa palm date fruit intake for four months on modulating epigenetic factors that were reported to be altered in autism.

Previous studies proved the effect of Ajwa dates fruit on different gene expressions resulting in the treatment and improvement of different diseases like hepatocellular carcinoma [60, 61] and breast cancer [62]. The consumption of Ajwa dates fruit is considered a safe and risk-free method of intervention [60-63]. Its intake was proved to be associated with the upregulation of dendritic cells in the upper gastrointestinal tract, proliferation and differentiation of systemic CD8+ T cells lymphocytes leading to improvement of the immune system [63] and acting as immunomodulatory [64] without causing any damage to the normal cell.

In the current study, we evaluated the effect of Ajwa palm date fruit intake for four months on the expression of two genes involved in epigenetic machinery, the first is *CHD8* which encodes an ATP-dependent chromatin remodeler. *CHD8* is not only documented to be altered in children with autism. *DNMT1* was previously reported to be decreased in the frontal cortex of the brains of ASD subjects [67], while another study documented its increase in the cerebellum of children with autism compared to neurotypical controls [68]. The differential expression of *DNMT1* between different tissues may explain the variation in its expression after receiving Ajwa dates fruit in our study. The variation in expression could also be attributed to a change in one or more of its regulatory factors, such as transcription factors or miRNAs that could be affected by one or more components of the Ajwa palm date fruit.

Next, we measured the change in the level of methylation in *LINE-1*, the promoter of *OR2L13* and *RELN*, and the *3'UTR of PRRT1*. *LINE-1* repetitive element is considered an index of global DNA methylation which is associated with genomic stability. Alteration of *LINE-1* was found in many diseases, including neurodevelopmental disorders [69]. a decreased level of *LINE*-1 methylation was found in cell lines derived from patients with severe language impairment displayed [70] and the blood of children with autism [44]. Also, decreased methylation of *PRRT1 3'UTR* was found in the temporal cortex and cerebellum of children with autism [71]. In our study, *LINE-1* and *3'UTR of PRRT1* showed significant change in their methylation density for both increase and decrease patterns after receiving Ajwa palm date fruit for four months on a daily basis (5 dates fruit/day), this variation in response could be explained by the involvement of other *DNMTs* which could be reported to be altered in ASD but also reported to regulate other ASD risk genes in human neurodevelopment and its loss contributes to ASD phenotype, suggesting that ASD risk genes are co-regulated and work as an ASD-associated regulatory network [65]. A previous study reported *CHD8* as one of the genes that exhibited down-regulation in ASD leukocytes compared to control subjects [66]. Therefore, we suggested that the up-regulation of *CHD8* will be associated with favourable outcomes for children with autism. We analyzed the expression level of *CHD8* in 31 patients before and after receiving Ajwa palm date fruits for four months to test the change in the *CHD8* expression level and if this change is associated with improving the ASD phenotype. However, the change in the expression was not significant.

The other gene we tested in our study is *DNMT1* which codes for one of the enzymes involved in DNA methylation and its primary role is in the maintenance of DNA methylation. We found a significant increase in 10 cases and a significant decrease in 20 cases, which supports our suggestion regarding the ability of nutrition to affect the expression of genes that are affected by the component of Ajwa palm date' fruits. This response in the variation of methylation status was also shown in the *OR2L13* promoter. This could be explained by the initial variation of methylation density of *OR2L13* promoter among cases before receiving the regimen; this suggestion is based on previous reports which mentioned that the promoter of *OR2L13* showed increased and decreased methylation levels in different studies [72]. Regarding *RELN*, an increased methylation level of *RELN* promoter was previously reported in the cerebellum [73], therefore, one of the aims of our study was the ability of our nutritional regimen to reduce the promoter methylation of RELN; we found a significant decrease of *RELN* methylation pattern in 23 subjects.

Considering that miRNAs are identified as one of the epigenetic factors, we analyzed the expression of two circulating miRNAs, *miR-146a* and *miR-164b*. *miR-146a* was found to impair ASD synaptic transmission and inhibit neuronal migration by targeting *MAP1B, GRIA3,* and *KCNK2* [74]. *miR-146a* and *miR-164b* were previously documented to be up-regulated in children with autism [75]. Therefore, we suggested that subjects who showed downregulation of *miR-146a* and *miR-146b* after the regimen would display improvement in ASD symptoms. In contrast to *miR-146b* which didn't show significant change in children with autism, a significant decrease of *miR-146a* was observed in 12 patients in response to the intake of Ajwa palm date fruit. The effect of Ajwa palm date fruit on miRNA expression could be due to its effect on the expression of transcription factors or

any component of epigenetic machinery that regulates miRNA expression.

It has been reported that autism is a complicated condition resulting from defects of several genetic pathways rather than resulting from dysfunction of particular genes [76]. It is a threshold disease model in which ASD phenotypes become apparent only after a certain burden of genetic risk alleles has been reached; the burden could apply within certain pathways. In addition, the role of environment, immunity, and nutrition and their interactions through genetic pathways can't be rolled out [76].

In response to Ajwa date fruit regular intake, we have studied the relation between the improvement of the observed clinical and laboratory data with the studied epigenetics parameters that showed only significant changes. It was found that the likelihood of having a decrease in *PRRT1* was significant with the improvement of GIT symptoms. Accordingly, we can recommend the intake of date fruits among practice guidelines for hemodialysis in Egypt for cases with renal affection [77, 78].

Previous studies showed that *PRRT1*, lower methylation (−9%) in a *DMR 3′ UTR* in the temporal cortex and cerebellum [79] was repeated by lower methylation (−7.8%) in the same region in the prefrontal cortex using the same platform [80]. This may be because we have inbuilt genetic redundancy and homeostatic mechanisms that could buffer or compensate for the loss of a specific gene within a pathway [81]. Nevertheless, such homeostatic mechanisms may not always fully compensate, leading to refined and maybe tissue-specific maladaptive phenotypic modification [82].

We found that the likelihood of having an increase of *RELN* was significant with the improvement of the presence of microorganisms. Previous studies found Lower levels of *RELN mRNA* in the frontal cortex and cerebellum in individuals with ASD [82]. Also, lower levels of RELN protein have been found in the brain and plasma of those with ASD [76]. Thus it is concluded that increased RELN is accompanied by some sort of improvement in children with autism according to internal homeostatic mechanisms and subtle tissue adaptation mechanisms [82].

Limitations of the study

This exploratory study has some limitations: It included a small number of participants with a wide age range (3- 12 years), so it could be considered a pilot study. This study is an interventional study with a single arm only without a comparative group; this restricts the inference of the benefits obtained with the use of Ajwa palm date fruit without knowing what it would have been in the absence of these dates fruit or the presence of other dates fruit. This trial was limited to the Ajwa palm date fruit that was imported from Saudi Arabia. It did not investigate the impact of other types of date fruit (including Egyptian types), a condition that might constrain the generalizability of the trial results. Due to the long intervention time, which is four months, some children were discounted from the study either because of the shift of the parents of the children to antioxidants or antibiotics or the incompliance because of the food selectivity of children with autism. Also, the authors relayed on parents' reports for the intake of their children of Ajwa dates fruits.

Strengths of the study

We believe that this pilot study accomplished our study objectives in a challenging way. One of the strengths of this exploratory study is that to our knowledge, it was the first to explore the epigenetic modifications of children with autism in response to Ajwa palm date fruit being a non-pharmacological and risk-free option for alleviation of autistic symptoms. The data provided preliminary results that may be considered as the base for a more extensive study population.

Conclusions

In response to Ajwa palm date fruit intake among ASD, the following was observed: A Significant decrease in the expression of *DNMT1* was shown in two-thirds of cases versus a significant increase in only one-third of cases. *LINE-1, OR2L13,* and *PRRT1* showed significant changes in their methylation density for both increase and decrease patterns. While the decreased pattern of RELN methylation status was significantly observed in all subjects. A significant decrease was observed in the mean of expression fold change of *MiR-146a* for almost half of the cases.

Recommendation

It is recommended to increase awareness about Palm date's value in general because of its content of minerals and polyphenols, which help in the improvement of many diseases like anemia [81], through community-based approaches that proved to be effective in raising awareness in Egypt, especially for women and their young children [83, 84]. Targeting mothers and their children early in life by improving their nutritional habits is very important for improving their cognitive function 85, 86]. Also, provision of the fortified diet with Ajwa at school age proved to be effective in improving their cognitive abilities[87- 89].

Therefore, future research of a large number of cases to elucidate the effect of Ajwa palm date fruit on the epigenetics of ASD holds promise for the therapeutic options that are based on epigenetic modifications, with potential effects on specific genes.

List of abbreviations

6-GSI	The Gastrointestinal Severity Index
AOAC	Association of Official Analytical Chemists
ABAS-3	Adaptive Behaviour Assessment System, Third Edition
ASD	Autism Spectrum Disorder
BMI	Body mass index
CARS	The Childhood Autism Rating Scale
cDNA	complementary DNA
CHD8	Chromodomain helicase DNA-binding protein 8
CON	Conceptual
DNMT1	DNA methyltransferase 1
DNMTs	DNA methyl transferases
DSM-V	Diagnostic and Statistical Manual of Mental Disorders (5th edition)
ELISA	Enzyme-linked Immune Sorbent Assay
FGID	Functional gastrointestinal behavior disorders
GAC	General Adaptive Composite
GIT	gastrointestinal tract
GPX1	Glutathione peroxidase
IQR	Interquartile range
LINE-1	Long interspersed nucleotide element-1
MDA	Malondialdehyde
MSP	Methylation-specific PCR
NRC	National Research Centre of Egypt
OR2L13	Olfactory receptor family 2 subfamily L member 13
PR	Practical
PRRT1	Proline Rich Transmembrane Protein 1
RELN	Reelin
SDU	Standard deviation units
SOC	Social
SOD	superoxide dismutase
SPSS	Statistical Package for the Social Sciences
TDF	total dietary fibers
VPA	Valproic Acid
WHO	World Health Organization

Ethics approval and consent to participate

The study was approved by the Medical Research Ethics Committee of the National Research Center (Approval Ethical Number: 19203). This study was registered at the US National Institutes of Health (ClinicalTrials.gov) # NCT04261595, with Protocol ID: 12060158. Parents of children with autism provided written informed consent during the initial visit. Confidentially of collected data was maintained. The conduct of the study complied with the International Ethical Guidelines for Biomedical Research Involving Human Subjects [80], and that information disclosure "Making sure patients understand" was guaranteed according to the recommendations of the Egyptian patients and guardians' perception about clinical informed consent as a preferred purpose for IC practices [81].

Competing interests

All authors report no conflict of interest. "No financial or non-financial benefits have been received or will be received from any party related directly or indirectly to the subject of this manuscript.

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References

1. Perfilyeva A, Bespalova K, Perfilyeva Y, Skvortsova L, Musralina L, Zhunussova G, et al. Integrative Functional Genomic Analysis in Multiplex Autism Families from Kazakhstan. Dis Markers. 2022; vol 2022, 1-26. doi: 10.1155/2022/1509994.
2. Ghandour RM, Sherman LJ, Vladutiu CJ, Ali MM, Lynch SE, Bitsko RH, et al. Prevalence and Treatment of Depression, Anxiety, and Conduct Problems in US Children. J Pediatr. 2019; 206: 256-267.e3.
3. Metwally A M, Helmy M A, Salah El-Din E M, Saleh R M, Abdel Raouf E R, Abdallah A M et al. (2023). National screening for Egyptian children aged 1 year up to 12 years at high risk of Autism and its determinants: a step for determining what ASD surveillance needs. BMC psychiatry 2023; 23(1): 471.
4. Courchesne E, Pierce K. Why the frontal cortex in autism might be talking only to itself: local over-connectivity but long-distance disconnection. Curr Opin Neurobiol 2005;15: 225-230.
5. Pardo CA, Eberhart CG. The neurobiology of autism. Brain Pathol 2007;17:434-447
6. M. Metwally, A., A. Helmy, M., Aboulghate, A. *et al.* The odds of having obesity in Egyptian children with autism spectrum disorders is higher than stunting compared to healthy developing

peers: a national survey. *BMC Pediatr* **24**, 465 (2024). https://doi.org/10.1186/s12887-024-04934-5
7. Loke YJ, Hannan AJ, Craig JM. The role of epigenetic change in autism spectrum disorders. Frontiers in neurology 2015; 6:107.
8. Tiffon C: The impact of nutrition and environmental epigenetics on human health and disease. International journal of molecular sciences 2018; 19(11).
9. Vijay A, Jha PK, Garg I, Sharma M, Ashraf MZ, Kumar B. Micro-rnas dependent regulation of dnmt and hif1alpha gene expression in thrombotic disorders. Sci Rep 2019; 9(1):4815.
10. Yao Q, Chen Y, Zhou X. The roles of micrornas in epigenetic regulation. Curr Opin Chem Biol 2019; 51:11-17.
11. Williams LA, LaSalle JM. Future prospects for epigenetics in autism spectrum disorder. Mol Diagn Ther 2022; 26(6):569-579
12. Anwar S, Raut R, Alsahli MA, Almatroudi A, Alfheeaid H, Alzahrani FM, et al. Role of Ajwa Date Fruit Pulp and Seed in the Management of Diseases through In Vitro and In Silico Analysis. *Biology*. 2022; 11(1):78.
13. Khalid S, Khalid N, Khan R S, Ahmed H & Ahmad A. A review on chemistry and pharmacology of Ajwa date fruit and pit. *Trends in Food Science and Technology* 2017; *63*: 60-69.
14. Wessels, I. Epigenetics and Minerals: An Overview. In: Patel, V., Preedy, V. (eds) Handbook of Nutrition, Diet, and Epigenetics. Springer, Cham 2017. Available from: https://doi.org/10.1007/978-3-319-31143-2_48-1
15. Frolinger T, Herman F, Sharma A, Sims S, Wang J, Pasinetti GM. Epigenetic modifications by polyphenolic compounds alter gene expression in the hippocampus. Biol Open. 2018; 4:7(10)
16. Hussein AM, Mahmoud SA, Elazab KM, Abouelnaga AF, Abass M, Mosa AAH, et al. Possible Mechanisms of the Neuroprotective Actions of Date Palm Fruits Aqueous Extracts against Valproic Acid-Induced Autism in Rats. Curr Issues Mol Biol 2023; 14;45(2):1627-1643.
17. American Psychiatric Association. Diagnostic and statistical manual of mental disorders-5 (DSM-V) (2013). Washington, DC: American Psychiatric Association
18. Schopler E, Reichler RJ, DeVellis RF, Daly K. Toward objective classification of childhood autism: childhood autism rating scale (CARS). J Autism Dev Disord 1980; 10(1):91–103
19. Chow S C, Shao J, Wang H, Lokhnygina Y. Sample size calculations in clinical research. chapman and hall/CRC; 2017. https://doi.org/10.1201/9781315183084. Accessed on 13 Jan 2019.
20. Accordini S. An introduction to sample size calculations in clinical trials. Epidemiology and Psychiatric Sciences 2007;16(4): 299-301.
21. Al Jaouni SK, Hussein A, Alghamdi N, Qari M, El Hossary D, Almuhayawi MS, et al. Effects of Phoenix dactylifera Ajwa on infection, hospitalization, and survival among pediatric cancer patients in a university hospital: a nonrandomized controlled trial. Integr Cancer Ther. 2019; 18(1): 1–9.
22. Marwat SK, Khan MA, Rehman F, BŚĂţ IU. aromatic plant species mentioned in the Holy Qura'n and Ahadith and their ethnomedicinal importance Pak J Nut.2009; 8: 1472-1479
23. Sahih al-Bukhari 5769. In-book reference: Book 76, Hadith 83. USC-MSA web (English) reference : Vol. 7, Book 71, Hadith 664.
24. AOAC. Association of Official Analytical Chemists. Official Methods of Analysis, Washington, D.C. 1990.
25. Nowatzk H, Ehrenberg J, Basico Gesellschaft. Extraction of water soluble substances from dates and production of amorphous sugar. 1976; Patent: German Federal Republic No. 2459353
26. Garcia O E, Infante R B and Rivera C J. Determination of total soluble and insoluble dietary fibre in two new varieties of Phaseolus vulgaris L. using chemical and enzymatic gravimetric methods. Food Chemistry 1997; 59(1): 171-174.
27. Fennema R. Owen. Food chemistry, 3rd Ed, Marcel Dekker, Inc. New Yourk1996.
28. Moriyama H, Yamasaki H, Masumoto S, Adachi K, Katsura N, Onimaru T. Rapid determination of vitamins A and E in serum with surfactant as a diluent by column-switching high-performance liquid chromatography. Journal of Chromatography A, 1999; 798(1-2): 125-130.
29. Evered D F. Determination of ascorbic acid in highly coloured solution with Nbromosuccinimide. J. Anal. 1960; 85: 515-517
30. Amidžić R, Brborić J, Čudina O, Vladimirov S. Rp-HPLC determination of vitamins, folic acid and B12 in multivitamin tablets. Journal of the Serbian Chemical Society. 2005; 70(10):1229-35.
31. Özcan M M. Determination of the mineral compositions of some selected oil-bearing seeds and kernels using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). GRASAS Y ACEITES 2006; 57(2): 211-218.
32. Dubois M, Gilles J K, Hamilton PA, Rebers PA and Smith F. Colorimetric method for determination of sugars and related substances, Analytical Chemistry, 1956; 28(3): 350-356.
33. Langemeier, J M and Rogers D E. Rapid Method for Sugar Analysis of Doughs and Baked Products. Cereal Chem 1995; 72(4): 349-351
34. Laurey S. Protein Structure Core Facility, UNMC, Omaha, NE 1997; 68198-4525
35. Kim D O, Jeong S W, and Lee C Y. Antioxidant Capacity of Phenolic Phyto- Chemicals from Variuos cultivars of Plums. Food Chemistry 2003; 81,321-326
36. Al-Farsi M A and Lee CY. Optimization of phenolics and dietary fibre extraction from date seeds. Food Chem. 2008; 108, 977–985.
37. Shaba E Y, Ndamitso M M, Mathew J T, Etsunyakpa M B, Tsado A N & Muhammad S S. Nutritional and anti-nutritional composition of date palm (Phoenix dactylifera L.) fruits sold in major markets of Minna Niger State, Nigeria. African Journal of Pure and Applied Chemistry 2015: 9(8): 167-174).

38. Assirey E A R. Nutritional composition of fruit of 10 date palm (Phoenix dactylifera L.) cultivars grown in Saudi Arabia. Journal of Taibah University for Science 2015; 9: 75-79
39. Khalid S, Ahmad A, Masud T, Asad M J & Sandhu M. Nutritional assessment of ajwa date flesh and pits in comparison to local varieties. Journal of Plant and Animal Sciences 2016; 26(4): 1072-1080
40. Al-Farsi M A & Lee C Y. Nutritional and functional properties of dates: A review. Critical Reviews in Food Science and Nutrition 2008; 48: 877-887.
41. Al-Shahib W & Marshall R J. The fruit of the date palm: Its possible use as the best food for the future? Int J Food Sci Nutr 2003; 54: 247-259.
42. Al-Shwyeh H A. Date Palm (Phoenix dactylifera L.) Fruit as Potential Antioxidant and Antimicrobial Agents. J Pharm Bioallied Sci. 2019;11(1):1-11
43. Ghnimi S, Umer S, Karim A, Kamal-Eldin A. Date fruit (Phoenix dactylifera L.): an underutilized food seeking industrial valorization. NFS J. 2017; 6:1–10.
44. Saeliw T, Permpoon T, Iadsee N, Tencomnao T, Hu VW, Sarachana T, et al. Line-1 and alu methylation signatures in autism spectrum disorder and their associations with the expression of autism-related genes. Sci Rep 2022; 12(1):13970.
45. Erhardt J. NutriSurvey for windows Copyright©. Jakarta: Seameo-Tropmed RCCN-University of Indonesia. 2007.
46. Hiernaux J, Tanner JM, Jarman S. Growth and physical studies. Human Biology: A guide to field methods. London: IBP. 1969.
47. Schneider C K, Melmed R D, Barstow LE, Enriquez FJ, Ranger-Moore J, Ostrem JA. Oral human immunoglobulin for children with autism and gastrointestinal dysfunction: a prospective, open-label study. J Autism Dev Disord 2006; 36(8):1053-64. doi: 10.1007/s10803-006-0141-y. PMID: 16845577.
48. Newby P K, Hu F B, Rimm E B, Smith-Warner S A, Feskanich D, Sampson L et al. Reproducibility and validity of the Diet Quality Index Revised as assessed by use of a food-frequency questionnaire. Am J Clin Nutr 2003;78(5):941-9. doi: 10.1093/ajcn/78.5.941. PMID: 14594780.
49. WHO Child Growth Standards (2006). Available from: http://www.who.int/childgrowth/psublications/technical_report_pub/en/. (Accessed 2022-06-29)
50. Harrison P L & Oakland T. Adaptive behavior assessment system manual (3rd ed.) Psychological Corporation 2015
51. Harrison P L, Oakland T. Adaptive Behavior Assessment System: Third Edition. In: Kreutzer, J.S., DeLuca, J., Caplan, B. (eds) Encyclopedia of Clinical Neuropsychology. Springer, Cham 2018. Available from: https://doi.org/10.1007/978-3-319-57111-9_1506
52. Tamm L, Day H A & Duncan A. Comparison of Adaptive Functioning Measures in Adolescents with Autism Spectrum Disorder Without Intellectual Disability. Journal of autism and developmental disorders 2022; 52(3):1247–1256. https://doi.org/10.1007/s10803-021-05013-9
53. James S J, Cutler P, Melnyk S, Hernigan S, Janak L, Gaylor DW, et al. Metabolic biomarkers of increased oxidative stress and methylation capacity in children with autism. Am J Clin Nutr 2004; 80(6):1611–1617
54. Chauhan A, Chauhan V. Oxidative stress in autism. Pathophysiology 2006;13(3):171–181
55. Almzaiel A J, Al-Ameri A M J, Tariq R. Role of IL-4 and Glutathione Peroxidase in Patients with Obstructive Lung Diseases. J. Kerbala Jorunal of Medicine, 2017, 10(2): 2714-2718.
56. Wheeler D L, Barrett T, Benson D A, Bryant K, Canese S H, Chetvernin D M, et al. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 2006; 34: D173-D180
57. Cole J R, Chai B, Marsh T L, Farris RJ, Wang Q, Kulam SA, et al. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 2003; 31: 442-443
58. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, et al. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res. 2000;28:E32.
59. Oakes, Christopher & La Salle, Sophie & Robaire, Bernard & Trasler, Jacquetta. (2006). Evaluation of a Quantitative DNA Methylation Analysis Technique Using Methylation-Sensitive/Dependent Restriction Enzymes and Real-Time PCR. Epigenetics : official journal of the DNA Methylation Society. 1. 146-52. 10.4161/epi.1.3.3392.
60. Siddiqui S, Ahmad, R., Khan, M.A. et al. Cytostatic and Anti-tumor Potential of Ajwa Date Pulp against Human Hepatocellular Carcinoma HepG2 Cells. Sci Rep 2019; 9: 245.
61. Khan F, Khan TJ, Kalamegam G, Pushparaj PN, Chaudhary A, Abuzenadah A, et al. Anti-cancer effects of Ajwa dates (Phoenix dactylifera L.) in diethylnitrosamine induced hepatocellular carcinoma in Wistar rats. BMC Complement Altern Med 2017; 17: 418.
62. Khan MA, Siddiqui S, Ahmad I, Singh R, Mishra DP, Srivastava AN, et al. Phytochemicals from Ajwa dates pulp extract induce apoptosis in human triple-negative breast cancer by inhibiting AKT/mTOR pathway and modulating Bcl-2 family proteins. Sci Rep 2021; 11: 10322.
63. Indarto D, Wasita B, Palupi PD. Immunomodulation of tahneeq method in IL-12 and CD8+ T-Lymphocyte, an in-vivo study in neonatal rats. Saudi Journal of Biological Sciences 2020; 1;27(10):2645-50.
64. Ahmad Mohd Zain MR, Abdul Kari Z, Dawood M. Bioactivity and pharmacological potential of date palm (Phoenix dactylifera L.) against pandemic COVID-19: a comprehensive review. Applied biochemistry and biotechnology. 2022;194(10):4587-624.
65. Cotney J, Muhle R A, Sanders S J Liu L, Willsey A J, Niu W, et al. The autism-associated chromatin modifier chd8 regulates other autism

risk genes during human neurodevelopment. Nature communications 2015; 6: 6404.
66. Pramparo T, Lombardo M V, Campbell K, Barnes C C, Marinero S, Solso S, et al. Cell cycle networks link gene expression dysregulation, mutation, and brain maldevelopment in autistic toddlers. Mol Syst Biol 2015; 11(12):841.
67. Zhubi A, Chen Y, Guidotti A, Grayson D R. Epigenetic regulation of reln and gad1 in the frontal cortex (fc) of autism spectrum disorder (asd) subjects. Int J Dev Neurosci 2017; 62: 63-72.
68. Keil K P & Lein P J. DNA methylation: A mechanism linking environmental chemical exposures to risk of autism spectrum disorders? Environ Epigenet 2016; 2(1).
69. Shpyleva S, Melnyk S, Pavliv O, Pogribny I, Jill James S. Overexpression of line-1 retrotransposons in autism brain. Mol Neurobiol 2018; 55(2):1740-1749.
70. Tangsuwansri C, Saeliw T, Thongkorn S, Chonchaiya W, Suphapeetiporn K, Mutirangura A, et al. Investigation of epigenetic regulatory networks associated with autism spectrum disorder (asd) by integrated global line-1 methylation and gene expression profiling analyses. PLoS One 2018; 13(7):e0201071.
71. Ladd-Acosta C, Hansen K D, Briem E, Fallin M D, Kaufmann W E, Feinberg A P. Common DNA methylation alterations in multiple brain regions in autism. Molecular psychiatry 2014; 19(8):862-871.
72. Forsberg S L, Ilieva M, Maria Michel T. Epigenetics and cerebral organoids: Promising directions in autism spectrum disorders. Translational psychiatry 2018; 8(1):14.
73. Zhubi A, Chen Y, Dong E, Cook E H, Guidotti A, Grayson D R. Increased binding of mecp2 to the gad1 and reln promoters may be mediated by an enrichment of 5-hmc in autism spectrum disorder (asd) cerebellum. Translational psychiatry 2014; 4(1):e349.
74. Wu X, Li W, Zheng Y. Recent progress on relevant micrornas in autism spectrum disorders. International journal of molecular sciences 2020; 21(16).
75. Talebizadeh Z, Butler M G, Theodoro M F. Feasibility and relevance of examining lymphoblastoid cell lines to study role of micrornas in autism. Autism Res 2008; 1(4):240-250.
76. Loke Y J, Hannan A J, Craig J M. The Role of Epigenetic Change in Autism Spectrum Disorders. Front Neurol. 2015; 26 (6):107.
77. Ahmed AMA,, Allam MF, Habil ES, Metwally AM, Ibrahiem NA, Radwan M, El Gaafary MM, et al. Development of practice guidelines for hemodialysis in Egypt. Published at Indian Journal of Nephrology (ISSN: 0971-4065), Volume 20 (4), October – December 2010; p: 193-202, DOI 10.4103/0971-4065.73450 https://www.scopus.com/record/display.uri?eid=2-s2.0-78650136027&origin=resultslist
78. Ahmed AMA, Allam MF, Metwally AM, Ibrahiem NA, Radwan M, El Gaafary MM, etal. Compliance with haemodialysis practice guidelines in Egypt. Eastern Mediterranean health journal (ISSN: 1020-3397, 1687-1634): WHO, Vol.19, (1): 2013; p:4-9, ISSN · 1687-1634, DOI 10.26719/2013.19.1.4
79. Ladd-Acosta C, Hansen K D, Briem E, Fallin M D, Kaufmann W E, Feinberg A P. Common DNA methylation alterations in multiple brain regions in autism. Mol Psychiatry 2014;19:862–71.
80. Nardone S, Sams D S, Reuveni E, Getselter D, Oron O, Karpuj M, et al. DNA methylation analysis of the autistic brain reveals multiple dysregulated biological pathways. Transl Psychiatry 2014; 4:e433.
81. Mitchell K J. The genetics of brain wiring: from molecule to mind. PLoS Biol 2007; 5:e113.
82. Zhubi A, Chen Y, Dong E, Cook EH, Guidotti A, Grayson D R. Increased binding of MeCP2 to the GAD1 and RELN promoters may be mediated by an enrichment of 5-hmC in autism spectrum disorder (ASD) cerebellum. Transl Psychiatry 2014; 4:e349
83. Metwally A M, Hanna C, Galal Y S, Saleh R M, Ibrahim N A, Labib N A. Impact of Nutritional Health Education on Knowledge and Practices of Mothers of Anemic Children in El Othmanyia Village – Egypt. Open Access Maced J Med Sci. 2020;30 ;8(E):458-65. DOI: 10.3889/oamjms.2020.4766 Available from: https://www.id-press.eu/mjms/article/view/4570
84. Metwally A M, Sallam S F, Alian K M, Abdel-Latif G A, Hasanin H M, Mawla M A, et al. Promoting weaning practices and growth of Egyptian infants by using communication for behavioral development approach. BMC Pediatrics 2022; 22: 689. Available from: https://doi.org/10.1186/s12887-022-03741-0
85. Metwally A M, Abdel-Latif G A, Mohsen A, El Etreby L, Elmosalami D M, Saleh R M, et al. Strengths of community and health facilities based interventions in improving women and adolescents' care seeking behaviors as approaches for reducing maternal mortality and improving birth outcome among low income communities of Egypt. BMC Health Services Research (ISSN: 1472-6963) 2020; 20: 592 Available from: https://doi.org/10.1186/s12913-020-05412-1
86. El Din E M S, Rabah T M, Metwally A M, Nassar MS, Elabd MA, Shalaan A, et al. Potential Risk Factors of Developmental Cognitive Delay in the First Two Years of Life. Open Access Maced J Med Sci. https://doi.org/10.3889/oamjms.2019.566. Volume 7, Issue 12, 30 June 2019, Pages 2024-2030
87. El-Din E M S, Elabd M A, Nassar M S. Metwally A M, Abdellatif G A, Rabah T M, et al. The Interaction of Social, Physical and Nutritive Factors in Triggering Early Developmental Language Delay in a Sample of Egyptian Children. OAMJMS 2019; 12: 7(17) 2767-2774. Available from: https://www.id-press.eu/mjms/article/view/oamjms. DOI: 10.3889/oamjms.2019.642
88. Metwally AM, El-Sonbaty MM, El Etreby LA, Salah El-Din EM, Abdel Hamid N, Hussien HA,

et al. Impact of National Egyptian school feeding program on growth, development, and school achievement of school children. World Journal of Pediatrics 2020; 16(4) 393-400. DOI 10.1007/s12519-020-00342-8. https://link.springer.com/article/10.1007/s12519-020-00342-8

89. Salah E M, Khalifa A G, Metwally A M, Abdel Hamid N, Hussien HA and Moneer Z M. The Impact Of School Snacks On Cognitive Function Of Primary School Children In Egypt" Journal of Applied Sciences Research 2012; 8(12): 5639-5650,
90. International Ethical Guidelines for Biomedical Research Involving Human Subjects. Geneva: CIOMS. 2016. https:// cioms. ch/ wp-conte nt/ uploa ds/ 2017/ 01/ WEB-CIOMS-Ethic alGui delin es. pdf
91. Metwally A M, Amer H A, Salama H I, Abd El Hady SI, Alam RR, Aboulghate A, et al. Egyptian patients'/guardians' experiences and perception about clinical informed consent and its purpose: Cross sectional study. PLoS ONE 2021; 16(6):e0252996. Available from: https:// doi. org/ 10. 1371/ journ al. pone. 02529 96.