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Original Article

Chlorella modulation of gut microbiota dysbiosis in patients with type-2 diabetes

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ABSTRACT — To investigate the effects of Chlorella alga on gut microbiota dysbiosis in type-2 diabetes (T2D). The stress perception of patients was also investigated. Chlorella (3 g/day) was administered to patients with T2D (n = 10) for a period of 30 days. Gut microbiota composition was analysed by 16S rDNA gene sequencing, and stress perception was evaluated using the perceived stress scale (PSS). A total of 13 phyla, 89 families, and 185 genera were identified from all faecal samples of patients with T2D, and Firmicutes and Bacteroidetes were the most dominant phyla among all samples. Chlorella decreased Bacteroidetes and increased Firmicutes. The proportions of the Akkermansia, Coprococcus, Dorea, Lachnospira, Phascolarctobacterium, and Ruminococcus generas increased, whereas the proportion of Paraprevotella, Prevotella, Klebsiella, and Sutterella decreased in the faeces of patients with T2D after Chlorella intake. Chlorella induced a significant reduction in perceived stress in patients with T2D, and better PSS scores negatively correlated with an increase in Akkermansia, Coprococcus, Dorea, Lachnospira, Phascolarctobacterium, and Ruminococcus and positively correlated with a decrease in Paraprevotella, Prevotella, Klebsiella, and Sutterella. Altogether, these results show the ability of Chlorella to positively modulate gut dysbiosis, leading to reduced stress perception in patients with T2D. Our findings contribute to the globally increasing search for new preventive and therapeutic strategies aimed at restoring the balance of the intestinal ecosystem.

Key words: Chlorella, Type-2 diabetes, Gut microbiome, Perceived stress scale

INTRODUCTION

The connection between gut microbiota and the pathogenesis of type-2 diabetes (T2D) and other hallmarks associated with metabolic syndromes is being increasingly recognised. Growing evidence indicates that dysbiosis is a key mechanism underlying the onset of low-grade chronic inflammation, leading to a rapid progression in the development of insulin resistance (Aydin *et al*., 2018; Cani, 2018; Scheithauer *et al*., 2020; Sharma and Tripathi, 2019). In addition, gut microbiota has emerged as an essential factor in the control of the gut–brain axis (Foster *et al*., 2017). This also provides evidence of microbiota composition changes in response to stressful situations (Molina-Torres *et al*., 2019), which is considered an important underlying condition in the development of T2D

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(Aschbacher *et al*., 2014; Kokoszka *et al*., 2009; Pouwer *et al*., 2005, 2010). In this context, research attempts to identify new preventive and therapeutic strategies aimed at restoring the balance of the intestinal ecosystem are increasing globally (Gagliardi *et al*., 2018).

Chlorella, a single-celled freshwater alga that contains all the nutrients necessary for human health (Matos *et al*., 2017; Rodriguez-Garcia and Guil-Guerrero, 2008; Vijayavel *et al*., 2007), is emerging as a prophylactic agent for the treatment of insulin resistance-related diseases (Torello *et al*., 2016b, 2016a; Vecina *et al*., 2014). Insulin resistance is related to a complex network of signalling pathways that lead to the chronic activation of serine kinases, thus promoting serine phosphorylation of IRS-1 (Shoelson *et al*., 2006; Solinas and Karin, 2010; Stumvoll *et al*., 2005) and diminished insulin-stimulated tyrosine phosphorylation of insulin receptor (IR), insulin receptor substrate (IRS), and protein kinase B (Akt) in the main insulin target tissues (Boura-Halfon and Zick, 2009). In this context, in a pioneering study (Vecina *et al*., 2014) we demonstrated that Chlorella prevents obesity-induced insulin resistance in high-fat diet-fed mice by modulating the insulin signalling pathway by increasing tyrosine phosphorylation of IR, IRS-1, and Akt in the main target tissues, which include the liver, skeletal muscle, and adipose tissue. Prevention of obesity-induced dyslipidaemia by reducing triglyceride, cholesterol, and free fatty acid levels along with the reestablishment of balance in the disturbed cytokine network were also observed in these mice (Torello *et al*., 2016a, 2016b).

Moreover, in a recent 12-month clinical study, we demonstrated that Chlorella ameliorates the inflammatory status and improves the quality of life of individuals with T2D and impaired glucose tolerance by restoring, to near-normal values, the altered levels of proinflammatory and anti-inflammatory cytokines, adipokines, and incretins (Martins *et al*., 2023). These findings corroborate previous studies showing the ability of the alga to suppress the expression of incretins (resistin) and related genes in the peripheral blood cells of patients with impaired glucose tolerance (Itakura *et al*., 2015). In this context, our present findings show the ability of Chlorella to contribute to the restoration of a balanced gut microbiota in patients with T2D, strengthen the evidence of the promising use of the alga as an adjuvant in the treatment of diseases related to insulin resistance and in a large number of multifactorial diseases that have recently been associated with functional and compositional alterations of the gut microbiome (Levy *et al*., 2017).

MATERIALS AND METHODS

Ethics

This study was approved by the Ethics Committee of the University of Campinas (UNICAMP) and was conducted in accordance with the ethical standards of the Declaration of Helsinki (CAAE 30981114.4.0000.5404). The study was registered in the Brazilian Registry of Clinical Trials (RBR-8rvrb5). Written informed consent was obtained from all the participants before starting the study.

Study design and participants

Patients with T2D were recruited from the Diabetes Group of the Community Health Centre (CECOM) of UNICAMP, Brazil. They were diagnosed with T2D by the clinical staff of the CECOM, according to the guidelines of the Brazilian Society of Diabetes (Sociedade Brasileira de Diabetes, 2014).To participate in the study, clinicians were required to be licenced in Campinas, Brazil, and have practiced medicine for an average of 12 years.

The inclusion criteria, as required by the Ethics Committee, were as follows: only patients with T2D who participated in the Diabetes Group of the CECOM were enrolled in the study; their biochemical parameters were within the normal reference range; participants would continue taking their prescribed treatment, which should be used for over 12 months at the onset of the investigations. The exclusion criteria were severe psychiatric illness, cancer, nephropathy, advanced cardiopathy, lung disease, type-1 diabetes, and a history of drug or alcohol abuse. Non-diabetic individuals were recruited from the clinical and administrative staff of the CECOM, and the requirements were good health and unaffected by T2D.

Chlorella tablets were administered for 30 days during breakfast, at 15 tablets/day (3.0 g). Evaluations were performed at baseline and 30 days after initiating Chlorella intake. The dosage regimen was determined based on previous clinical trials (Azocar and Diaz, 2013; Merchant and Andre, 2001; Miyazawa *et al*., 2013b; Mizoguchi *et al*., 2008; Nagayama *et al*., 2014; Nakano *et al*., 2007, 2010; Noguchi *et al*., 2014; Panahi *et al*., 2012).

The Chlorella tablets were composed of 100% Chlorella powder (*Clhorella vulgaris* CK-5) that was cultured and manufactured/supplied by Chlorella Industry Co. Ltd., Japan. According to prior analyses, each tablet was composed of the following (/100g): protein, 57.4 g; lipid, 8.6 g; total carbohydrates, 21.8 g; calories, 394 kcal; iron, 95 mg; saturated fatty acid, 1.67 g; monounsaturated fatty acid, 1.60 g; polyunsaturated fatty acid, 4.09 g; omega-3, 1.49 g; omega-6, 2.0 g dietary fibre, 11 g; vitamin B_1, 1.8 mg; vitamin B_2, 5 mg; vitamin B_{12}, 500 µg;

vitamin C, 60 mg; folic acid, 2500 µg; biotin, 300 µg; α-tocopherol, 30 mg; vitamin K_1, 3000 µg; potassium, 1000 mg; magnesium, 350 mg; chlorophylls, 3.2 g; lutein, 265 mg; β-carotene, 99.7 mg; zeaxanthin, 28 mg; α-carotene, 13.9 mg; linoleic acid, 2.0 g; and α-linoleic acid, 1.9 g (Maruyama *et al*., 2018; Miyazawa *et al*., 2013a; Vecina *et al*., 2014).

Sample collection and analytical methods

Faecal samples were obtained from patients using sterile gloves and seat protectors (ColOff®, São Paulo, Brazil) at baseline and after 30 days of Chlorella intake. Faeces were collected in sterile stool collection tubes with a stool DNA stabiliser (Stratech, Ely, United Kingdom, cat. 1038111300) to avoid contamination and ensure the integrity and quality of the bacterial DNA. All samples were stored in sterile tubes at −80°C until use.

The microbiota profile was determined by 16S rRNA gene sequencing. Genomic DNA was extracted from faecal samples using the PSP Spin Stool DNA Plus Kit (Stratech, cat. 1038110300), according to the manufacturer's protocol. DNA concentration and quality in the samples were determined by agarose gel electrophoresis using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The V3 and V4 regions of the 16S rRNA gene were amplified using the following primers: 5′−*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG***CCTACGGGNGGCWGCAG**−3′ and 5-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG***GACTAC VGGGTATCTAA TCC**−3′. The portion in italics corresponds to the adapter Nextera®, and the sequences in bold are the widely conserved V3-341 and V4-785 initiators. The taxonomic composition of the bacterial communities was obtained by analysing the V3 and V4 regions of the 16S rRNA gene using the Illumina MiSeq platform. The DNA sequencing libraries were constructed according to the manufacturer's instructions (Illumina, San Diego, CA, USA), following the protocol of Caporaso *et al*. (2012). Sequencing was performed using the Illumina MiSeq equipment in the Laboratory of Molecular Genetics of the School of Medical Sciences of the University of Campinas, Brazil. The experiment was designed to obtain overlapping fragments paired with 300 base pairs and generated an output of 100.000 lectures per sample. The following computational analysis tools were used in the analysis: FastQC, QIIME, and OTU identification, with Greengenes reference OTUs (version 13.8).

Clinical measurements

A questionnaire was administered to evaluate demographic and clinical characteristics, pharmacotherapy, habits, and use of drugs or alcohol. Body mass index was calculated according to the guidelines of the Brazilian Association for the Study of Obesity and Metabolic Syndrome (ABESO, 2016)

Perceived stress scale evaluation

The Brazilian standardised version of the 14-question perceived stress scale (PSS) (Luft *et al*., 2007) was used to measure the degree to which life situations were considered stressful. It is an open access instrument that assesses the extent to which respondents perceive life as unpredictable, uncontrollable, and overloaded (Cohen *et al*., 1983). The PSS contains general rather than event-specific items and is designed for use in community populations with at least a junior high school education. The questions in the PSS ask about feelings and thoughts during the last month, and in each case, respondents were asked how often they felt a certain way. The scale contains 14 questions with a Likert-style response, with the final sum ranging from 0 to 56. Each question has five possible answers, with values of 0 = never, 1 = almost never, 2 = sometimes, 3 = almost always, and 4 = always. The stress levels were divided into three categories based on the following scores: low, 0–15; average, 16–20; high/very high, > 21.

Statistical analysis

The demographic and clinical characteristics are expressed as frequencies and percentages for categorical variables. For continuous variables, we used the mean and SD. The analysis of the gut microbiota was performed using the Explicet v2.10.5 (Robertson *et al*., 2013) and LEfSe (Segata *et al*., 2011) software. The Wilcoxon rank-sum test was used to compare bacterial abundance at different taxonomic levels. Significant statistical differences in the sample groups were tested using a multivariate non-parametric analysis of variance. LDA Effect Size (LEfSe), an algorithm for high-dimensional biomarker discovery, was used to identify taxa that discriminated microbiota profiles according to diet and treatment. Differences were considered statistically significant at $p < 0.05$. GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses.

RESULTS

Population

As shown in Table 1, in the T2D group (n = 12), 92% were female, with a mean age of 60 ± 8 years. The prevalence of overweight/obesity was 58%, and 67% of the cohort had hypertension. Seventeen percent used insu-

Table 1. Characteristics of the studied population.

Characteristics	Type-2 diabetes (T2D)	
	n = 12	Percentage
Sex		
Female	11	92
Male	1	8
Age (years)		
< 60	5	42
≥ 60	7	58
Age mean ± SD (Minimum–maximum)	60 ± 8	43–72
Body mass index (kg/m^2)		
Normal < 25	5	42
Overweight/obese ≥ 25	7	58
Occupation		
Employed	8	67
Retired	4	33
Drug use		
None	1	8
Metformin only	2	17
Hypoglycaemic therapy	7	58
Insulin plus metformin	2	17
Hypertension		
Yes	8	67
No	4	33
Physical activity		
Yes	10	83
No	2	17

lin, 58% received hypoglycaemic therapy (metformin plus glibenclamide, gliclazide, glimepiride, pioglitazone, or sitagliptin), 17% used metformin alone, and 8% received a treatment based on lifestyle changes. Sixty-six percent were employed, and 33% were retired. As shown in Table 2, no significant ($p > 0.05$) changes in biochemical parameters were observed in any of the individuals throughout the study.

Gut microbiota (taxonomy-based comparisons of faecal microbiota at the phylum and genus level)

Over 96% of the sequences of phyla detected in the faecal samples of the patients with T2D consisted of Firmicutes (45%), Bacteroidetes (42%), Proteobacteria (6.8%), and Verrucomicrobia (2.8%) (Fig. 1A), and the remaining sequences consisted of Actinobacteria, Cyanobacteria, Elusimicrobia, Fusobacteria, Lentisphaerae, Proteobacteria, Spirochaetes, Synergistetes, TM7, and Tenericutes (data not shown). Chlorella intake significantly modulated the bacterial abundance of the four more abundant phyla by increasing the approximate proportions of Firmicutes to 51% and of Verrucomicrobia to 3.6% and reducing the approximate proportions of Bacteroidetes to 37% and of Proteobacteria to 5.1% ($p < 0.05$) (Fig. 1B).

At baseline, 185 genera were identified in the faecal samples of patients with diabetes. Of these, the following 10 genera were differentially abundant ($p < 0.05$) after Chlorella intake: in the Firmicutes phylum, increased relative abundance was observed in five genera: Coprococcus (↑1.3-fold), Dorea (↑1.4-fold), Lachnospira (↑1.5-fold) (Lachnospiraceae family), Phascolarctobacterium (↑1.6-fold) (Veillonellaceae family), and Ruminococcus (↑2.8-fold) (Ruminococcaceae family) (Fig. 2A). Increased relative abundances were also observed in the Verrucomicrobia phylum and Akkermansia genera (↑2.9-fold) (Verrucomicrobiaceae family) (Fig. 2B). In the Bacteroidetes phylum, decreased relative abundances (↓10-fold) were observed in two genera: Prevotella (Prevotellaceae family) and Paraprevotella (Paraprevotellaceae family) (Fig. 2C). Decreased relative abundances were also observed in two genera of the Proteobacteria phylum: Sutterella (↓0.7-fold) (Alcaligenaceae family) and Klebsiella (↓5-fold) (Enterobacteriaceae family) (Fig. 2D).

Perceived stress - PSS score

As shown in Fig. 3A, after 30 days of Chlorella intake, there was a significant reduction in the degree of perceived stress in patients with T2D (28 ± 6 vs. 17 ± 6; $p = 0.0033$). Notably, the percentage of individuals in the range of high/very high stress reduced from 83% to 25%, whereas in the range of low and average stresses, the increases varied from 0% to 34% and from 17% to 41%, respectively (Fig. 3B).

Correlations between gut microbiome and changes in stress perception

As shown in Fig. 4, correlations between gut microbiota dysbiosis and stress perception were found after Chlorella intake. Notably, the better PSS scores negatively correlated with increasing proportions of Akkermansia, Coprococcus, Dorea, Lachnospira, Phascolarctobacterium, and Ruminococcus (Fig. 4A), and positively correlated with decreasing proportions of Paraprevotella, Prevotella, Klebsiella, and Sutterella (Fig. 4B).

DISCUSSION

The role of the gut microbiota in T2D and other metabolic disorders is gaining increasing importance (Aydin *et al*., 2018; Delzenne *et al*., 2020; Gurung *et al*., 2020). A common mechanism in dysbiosis seems to be related to the leakage of inflammatory mediators from metabolites of gut microbes into the systemic circulation, thus compromising the integrity of the gut barrier, leading to low-grade chronic inflammation and insulin resistance (Harsch

Table 2. Biochemical parameters (mean ± SD) in patients with type-2 diabetes (T2D) after Chlorella intake.

Parameters	*Reference values*	Baseline	30 days	*P* value*
Glucose (mg/dL)	*< 99*	140 ± 59	125 ± 44	NS
Insulin (uUI/mL)	*3.2–16.4*	22 ± 20	14 ± 9	NS
HOMA-IR	*2.71*	9 ± 12	2 ± 3	NS
HbA1c (%)	*< 6.4*	7.8 ± 2.5	7.6 ± 2.0	NS
Cholesterol (mg/dL)	*< 200*	180 ± 39	167 ± 35	NS
LDL (mg/dL)	*100–129*	103 ± 32	83 ± 22	NS
HDL (mg/dL)	*men ≥ 40* *women ≥ 50*	54 ± 16	50 ± 15	NS
Triglycerides (mg/dL)	*< 150*	114 ± 66	143 ± 81	NS
ALT (U/L)	*men < 50* *women < 35*	21 ± 9	16 ± 8	NS
AST (U/L)	*men < 50* *women < 35*	20 ± 4	19 ± 4	NS
Iron (ug/dL)	*men 70–180* *women 60–180*	88 ± 27	75 ± 24	NS
Ferrintin (ng/mL)	*men 30–400* *women 13–150*	209 ± 157	197 ± 142	NS
C-reactive protein (mg/L)		4.2 ± 4.0	4.7 ± 5.4	NS

*As determined by repeated measures ANOVA followed by Bonferroni's multiple comparison test (*P* value represents the difference in the patients' biochemical parameters between baseline and 30 days after Chlorella intake). NS, non-significant.

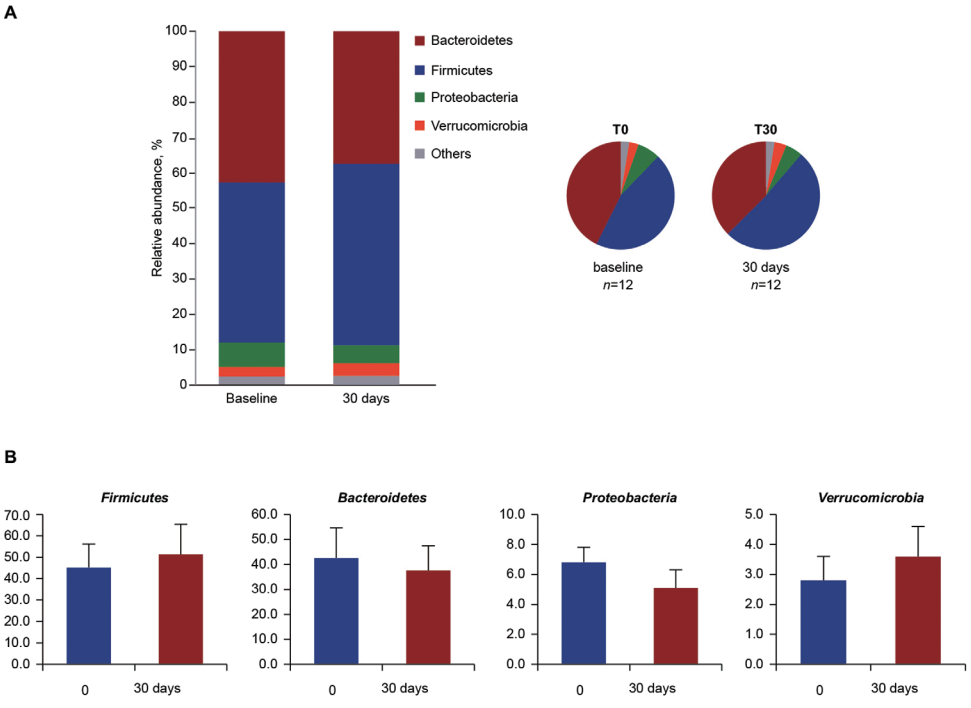

Fig. 1. Phyla distribution of gut microbiota in patients with type-2 diabetes (T2D) (n = 12) at baseline and after 30 days of Chlorella intake. (**A**) Distribution of the phyla as a percentage of the total number of identified 16S rDNA sequences in the groups. (**B**) Relative abundance of observed sequences (%) in the phyla *Bacteroidetes, Firmicutes, Proteobacteria* and *Verrucomicrobia* significantly differ after 30 days of Chlorella intake. *p < 0.05; Wilcoxon test.

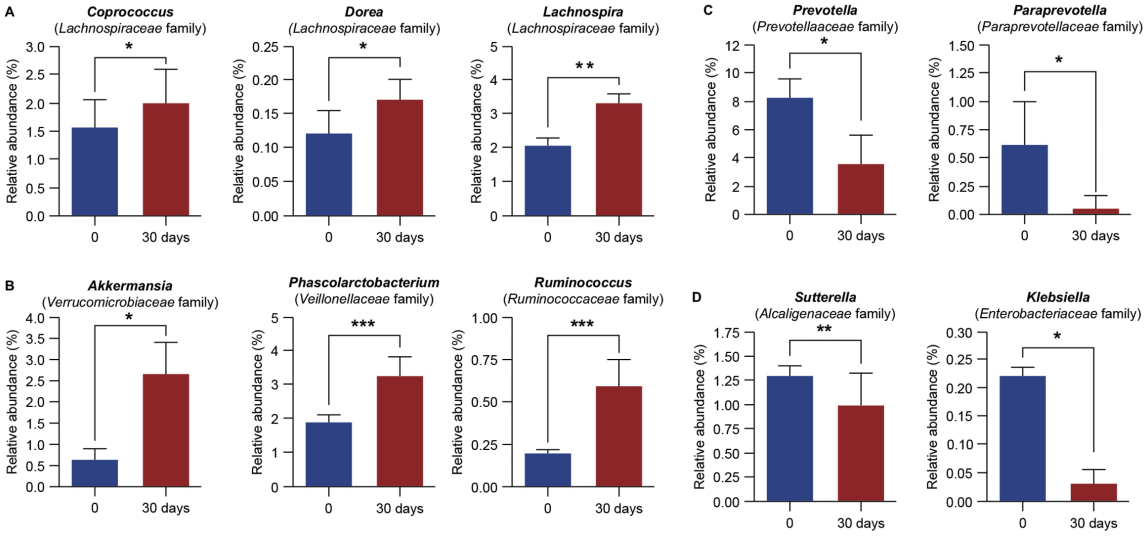

Fig. 2. Relative abundance of observed sequences (%) from bacterial genera with significant changes after Chlorella intake determined in gut microbiota of patients with type-2 diabetes (T2D) (n = 12) at baseline and after 30 days. *p < 0.05; Wilcoxon test.

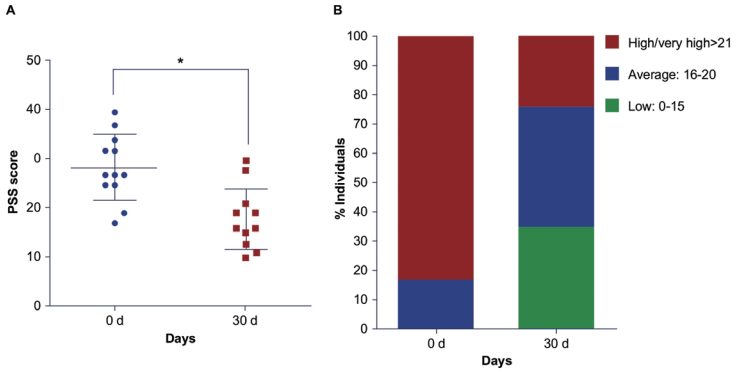

Fig. 3. Chlorella intake (**A**) reduces the perceived stress scale (PSS) score of patients with type-2 diabetes (T2D) (n = 12) after 30 days. (**B**) Percentage of patients with T2D divided into three score categories (low: 0–15; average: 16–20; high/very high > 21) of PSS at baseline and after 30 days of Chlorella intake. *p < 0.05; Wilcoxon test.

and Konturek, 2018; Upadhyaya and Banerjee, 2015). Diet is considered one of the most important influences on gut microbiota homeostasis. Attempts to use a diet-based approach to correct dysbiosis have been successfully developed, with the advantage that significant changes in the function and composition of microbiota can occur as early as 48 h diet change. However, only long-term diets can reach the magnitude required to relocate enterotypes and produce the necessary changes to shape the microbiota composition (Aydin *et al*., 2018; Gagliardi *et al*., 2018; Upadhyaya and Banerjee, 2015; Valdes *et al*., 2018). In this context, in a recent 12-month study in individuals with T2D and pre-diabetes, we demonstrated the ability of Chlorella to restore near-normal values of altered cytokine, adipokine, and incretin levels and significantly improve quality of life. These findings reveal the adaptogen activity of the alga on inflammatory pathways and highlight its promising use as a complementary alternative in the treatment of diseases related to insulin resistance (Martins *et al*., 2023).

Our present results corroborate studies in the literature that have shown a decreased abundance of certain universal butyrate-producing bacteria and an increased abundance of opportunistic pathogens in patients with

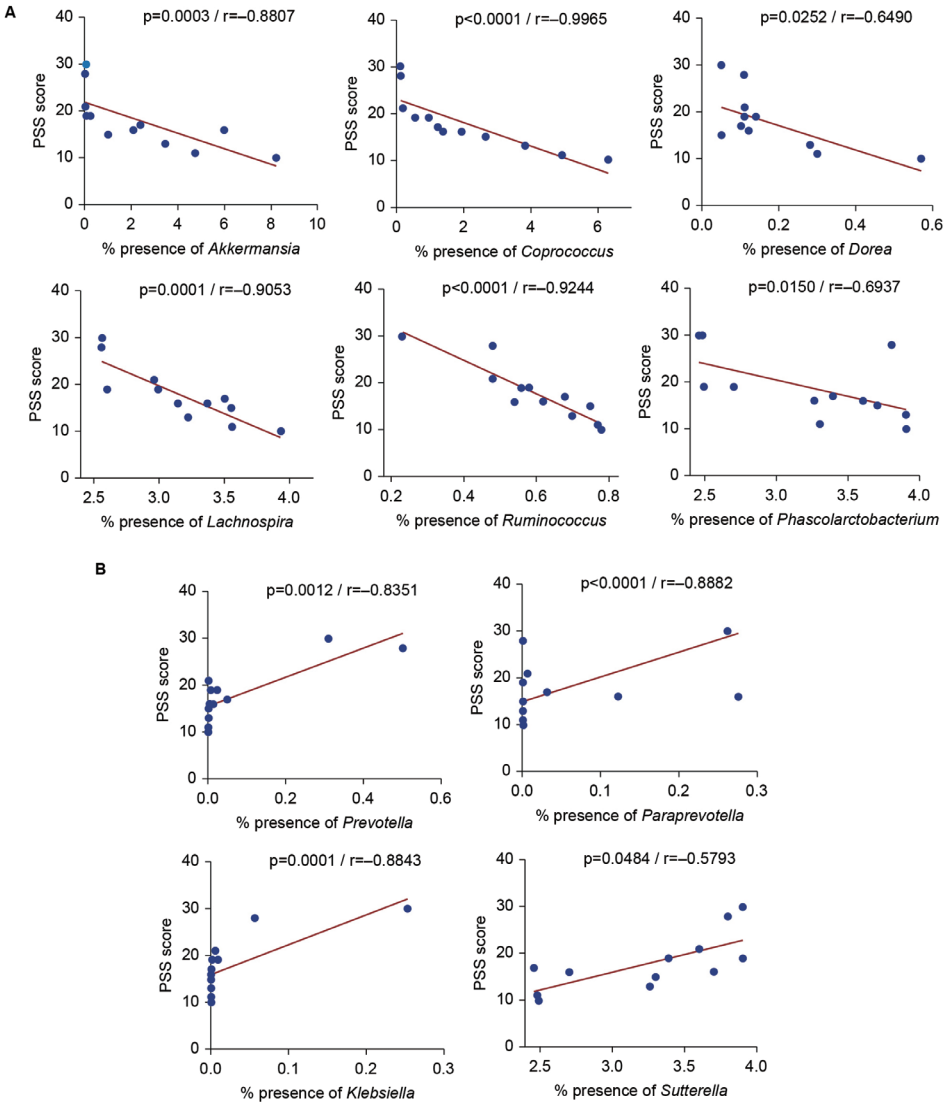

Fig. 4. Correlations of perceived stress scale (PSS) score and percentage of presence of (**A**) *Akkermansia*, *Coprococcus, Dorea*, *Lachnospira*, *Ruminococcus, Phascolarctobacterium,* and (**B**) *Prevotella*, *Paraprevotella*, *Klebsiella*, and *Sutterella* in patients with type-2 diabetes (T2D) (n = 12) after 30 days of Chlorella intake. The values of "p" and "r" are represented in the graphs, Spearman test.

T2D (Qin *et al*., 2012). At baseline, we observed that, in addition to the six main phyla that are usually dominant in the gut microbiota (Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Verrucomicrobia, and Proteobacteria) (Human Microbiome Project Consortium, 2012), seven other phyla (Cyanobacteria, Elusimicrobia, Lentisphaerae, Spirochaetes, Synergistetes, TM7, Tenericutes) were also detected, at a much lower scale, in the stools of patients with T2D. Firmicutes was the most prevalent phylum, followed by Bacteroidetes, and both accounted for over 80% of the total gut microbiota.

After Chlorella treatment, 10 out of the 185 genera identified in this study were differentially abundant ($p < 0.05$), and changes were detected in the following four phyla: Firmicutes, Verrucomicrobia, Bacteroidetes, and Proteobacteria. Notably, upmodulating effects of the alga were identified in both the Firmicutes and Verrucomicrobia phyla. The Firmicutes phylum was represented by the genera Coprococcus, Lachnospira, Ruminococcus, Dorea, and Phascolarctobacteria, and the Verrucomicrobia phy-

lum was represented by the genus Akkermansia. Conversely, downmodulating effects were produced on the Bacteroidetes phylum, represented by the Prevotella and Paraprevotella genera, and on the Proteobacteria phylum, represented by the genera Sutterella and Klebsiella.

These findings are relevant because Firmicutes are recognised as the predominant producers of short chain fatty acid (SCFA) metabolites derived from the microbial fermentation of dietary fibres, which are important fuel for the intestinal epithelial cells that maintain intestinal homeostasis and strengthen the gut barrier function. In this context, one noteworthy feature of Chlorella is its rich content of carbohydrates and dietary fibres, as mentioned previously. SCFAs, especially butyrate, are considered the major source of nutrition for colonic epithelial cells, which modulate the surrounding microbial environment and directly interact with the host immune system (Parada Venegas *et al*., 2019). The beneficial activities of SCFAs are related to improved glucose homeostasis and insulin sensitivity, reduced inflammation and insulin resistance, and increased secretion of glucagon-like peptide-1 (GLP-1), a hormone with insulinotropic activity that regulates glucose and lipid metabolism and increases intestinal motility (Campbell and Drucker, 2013). This can potentially improve metabolic control in T2D (Canfora *et al*., 2015; Müller *et al*., 2019; Puddu *et al*., 2014). In this context, clinical findings point to the potential role of SCFAs as novel therapeutic agents for preventing and counteracting obesity and other disorders related to glucose metabolism and insulin resistance (Canfora *et al*., 2015; Chambers *et al*., 2018; Tang and Li, 2021; Qin *et al*., 2012).

As for the Firmicutes phylum genera that presented increased abundance after Chlorella intake, the members of the Lachnospiraceae family are among the main producers of SCFAs and have repeatedly been shown to produce beneficial metabolites for the host and positively associate with T2D in both humans and animal models (Kameyama and Itoh, 2014; Vacca *et al*., 2020; Qin *et al*., 2012). Clinical and experimental studies (Gomez-Arango *et al*., 2016) have shown that an increased abundance of the genus Coprococcus positively associated with increased levels of gastrointestinal GLP-1. These findings corroborate previous studies showing that non-digestible fermentable dietary fibres have an antidiabetic effect that requires a functional GLP-1 receptor, thus emphasising the therapeutic potential of enhancing the endogenous secretion of this hormone for T2D treatment (Cani *et al*., 2006). In this context, our recent 12-month study on the effects of Chlorella in patients with diabetes and pre-diabetes has shown the ability of the alga to reduce inflammation, increase GPL-1 release, and consequently decrease the levels of glucagon (Martins *et al*, 2023), which is a counter-regulatory hormone that neutralises insulin actions (Gaisano *et al*., 2012; Habegger *et al*., 2010). These results are in agreement with experimental studies showing that diabetic animals fed a Chlorella-supplemented diet showed reduced fasting glucagon levels similar to those of exendin-4, a GLP-1 receptor agonist resistant to dipeptidyl peptidase-IV (Jeong *et al*., 2009). The ability of Chlorella to increase the Dorea genus is in line with previous findings showing that a fibre-rich diet is effective in counteracting the decrease in Dorea in patients with T2D (Candela *et al*., 2016). The genus Phascolarctobacterium has been shown to be abundantly colonised in the human gut and positively correlated with positive mood in humans, thus suggesting a critical role for this species in the dynamic balance of the gut microbiota (Wu *et al*., 2017). The Ruminococcus genus has been shown to assist gut epithelial cells in absorbing sugars, and its abundance has been positively associated with T2D (Gurung *et al*., 2020).

Akkermansia muciniphila is a mucin-degrading bacterium that resides in the mucus layer and represents 1%–4% of the faecal microbiota of healthy people. Members of the genus Akkermansia have been suggested as biomarkers for a healthy gut due to their ability to link to intestinal health and improve metabolic conditions in patients with obesity and T2D. The presence of viable *A. muciniphila* within the mucus layer is a crucial mechanism in the control of host mucus turnover, which plays a key role in the pathophysiology of obesity, T2D, and metabolic inflammation (Everard *et al*., 2013). Furthermore, its ability to increase the thickness of the mucus layer restores the intestinal barrier function and helps normalise metabolic endotoxaemia and adipose tissue metabolism (Belzer and de Vos, 2012; Derrien *et al*., 2017). In agreement with animal studies, a negative association between the abundance of this bacterium and T2D has been reported in human studies (Greer *et al*., 2016; Gurung *et al*., 2020; Zhang *et al*., 2013). A lower abundance of this bacterium was found in patients with T2D, in association with low-grade inflammation and gut permeability disruption (Pascale *et al*., 2019). A beneficial effect of *A. muciniphila* in the prevention and amelioration of metabolic disorders and better clinical outcomes has been shown in the majority of studies (Dao *et al*., 2016; Xu *et al*., 2020).

Another important feature of *Akkermansia muciniphila* is the induction of anti-inflammatory cytokines, such as IL-10, thus maintaining intestinal homeostasis (Scheithauer *et al*., 2020) and contributing to the improvement of glucose metabolism. In this context, in our 12-month study of the effects of Chlorella in patients

with T2D, the alga increased the levels of both anti-inflammatory proteins, adiponectin and IL-10 (Martins *et al.*, 2023). This finding is relevant because reduced expression of these proteins is related to increased insulin resistance (Calle and Fernandez, 2012). Adiponectin regulates insulin signalling by increasing the translocation of glucose transporter type-4 (GLUT-4) to the membrane, thus enhancing the peroxisome proliferator-activated receptor (PPAR) α/γ-mediated pathways in the liver and skeletal muscle through the selective activation of anti-inflammatory cytokines, such as IL-10 (Dasari and Raghunath, 2018; Itakura *et al*., 2015). Additional studies have demonstrated the ability of Chlorella to produce a dual activation of PPAR-α/γ (Chou *et al*., 2008) and increase the expression of GLUT-4, which allows for an influx of glucose in the liver and muscle (Lee and Kim, 2009).

The effects of Chlorella on reducing the abundance of gram-negative bacteria are relevant because increased levels of these bacteria in the gut correlate with high circulating lipopolysaccharide, which is considered a triggering factor for high insulin resistance in T2D (Cani *et al*., 2007). Prevotella spp. has been shown to be associated with gut inflammation, favouring neutrophil recruitment, systemic dissemination of inflammatory mediators, increased intestinal permeability, and translocation of bacterial products, which amplify and promote systemic inflammation (Larsen, 2017). The Paraprevotella genus was reported to have significantly increased relative abundance in the gut microbiota of patients with T2D with chronic kidney disease, compared with controls (Salguero *et al*., 2019). Overgrowth of Klebsiella leads to pneumonia, diarrhoea, and urinary tract infection and usually indicates gut dysbiosis. This can lead to a variety of serious chronic diseases, such as colitis, Crohn's disease, and ankylosing spondylitis (Ebringer *et al*., 2007; Garrett *et al*., 2010). The Sutterella genus has been frequently associated with human diseases such as autism, Down syndrome, and inflammatory bowel disease (Biagi *et al*., 2014; Hiippala *et al*., 2016; Lavelle *et al*., 2015; Wang *et al*., 2013).

Therefore, it seems that the ability of Chlorella to increase the abundance of putatively good bacteria and reduce the gram-negative bacterial content of the digestive tract could be viewed as a positive effect of the alga. This corroborates our previous findings of the anti-inflammatory effects of the alga in patients with pre-diabetes and T2D (Martins *et al*., 2023).

The ability of Chlorella to increase SCFA production might also be related to its rich iron content (Vecina *et al*., 2014). Iron is required for most gut bacteria, and many of them have active iron transport systems and other mechanisms to scavenge this essential trace element (Andrews *et al*., 2003). Experimental studies in iron-deficient rats showed significantly lower concentrations of caecal butyrate and propionate, together with a decrease in major butyrate-producing bacteria. Repletion of iron significantly increased caecal butyrate concentrations. Therefore, iron deficiency in the gut microbiota increases the risk of gut inflammation and colonic neoplasia due to markedly lower amounts of colonic butyrate (Dostal *et al*., 2012).

The rich content of carotenoids, especially lutein, present in Chlorella might be a key factor in the homeostatic modulation of inflammatory pathways by algae. Studies in healthy volunteers showed that a single 6 g dose of Chlorella increased the fasting serum lutein concentration by 1.5-fold, and these levels were maintained for over a 3-day period (Shibata and Hayakawa, 2009). In another study, a daily dietary intake of 9 g of Chlorella for 2 months led to a four-fold increase in the concentration of lutein in erythrocytes. In addition, one month after cessation of Chlorella intake, lutein returned to basal levels, suggesting that daily Chlorella intake is effective for improving and maintaining erythrocyte lutein concentration in humans (Miyazawa *et al*., 2013a). This is relevant because human beings are unable to synthesise carotenoids from endogenous precursors and thus have to acquire them from the diet (Eggersdorfer and Wyss, 2018).

Carotenoids prevent the development of the inflammatory response and insulin resistance, mainly through their effect on intracellular signalling pathways by blocking the translocation of NF-κB to the nucleus and inhibiting the downstream production of inflammatory cytokines (Martins *et al.*, 2023). This is also a well-recognised mechanism of butyrate's anti-inflammatory activity (Vinolo *et al*., 2011). We corroborated these findings in obese/T2D mice, which showed the ability of Chlorella to restore the increased levels of proinflammatory markers and reduced levels of anti-inflammatory cytokines to normal values, in association with its ability to inhibit the activation of NF-κB signalling pathway by regulating IRS-1 function. This occurs through a homeostatic balance between 'positive' IRS-1 tyrosine phosphorylation versus 'negative' IRS-1 serine phosphorylation, in association with a homeostatic modulation of cytokine production (Torello *et al*., 2016a; Vecina *et al*., 2014).

Insulin resistance is an important pathophysiological mechanism that predicts the progression of T2D (Donath and Shoelson, 2011). In many cases, insulin resistance is associated with a complex network of signalling pathways, including the reduced insulin-stimulated tyrosine phosphorylation of IR and IRS and Akt serine phos-

phorylation in the main target tissues of insulin, such as the liver, skeletal muscle, and adipose tissue (Boura-Halfon and Zick, 2009). The mechanism responsible for these alterations is the chronic activation of several serine kinases, such as c-Jun N-terminal kinase and inhibitor of kappa β kinase, which gives rise to obesity-induced insulin resistance by promoting serine phosphorylation of IRS-1 (Shoelson *et al*., 2006; Solinas and Karin, 2010; Stumvoll *et al*., 2005).Thus, several studies have recommended that the phosphorylation levels of IRS-1 on serine residue 307 (IRS-1 ser307) in rodents or serine residue 312 (IRS-1 ser312) in humans could be used as an insulin resistance marker (Shoelson *et al*., 2006; Solinas and Karin, 2010; Stumvoll *et al*., 2005).

Stress has long been suspected to be a key factor in the development of T2D (Kokoszka *et al*., 2009; Menninger, 1935; Pouwer *et al*., 2005, 2010; Slawson *et al*., 1963; Willis, 1674). Clinical studies have shown that the synergistic combination of stress and high consumption of high-fat/sugar foods is associated with enhanced vulnerability to diet-related metabolic risks, such as abdominal adiposity, insulin resistance, and oxidative stress (Aschbacher *et al*., 2014). In addition, mounting evidence has linked stress and depression-like behaviour to microbial dysbiosis (Carabotti *et al*., 2015; Inserra *et al*., 2018; Zheng *et al*., 2016). In our study, Chlorella treatment significantly reduced perceived stress in patients with T2D. After 30 days of Chlorella intake, the better PSS scores negatively correlated with increasing proportions of Akkermansia, Coprococcus, Dorea, Lachnospira, Phascolarctobacterium, and Ruminococcus, and positively correlated with decreasing proportions of Paraprevotella, Prevotella, Klebsiella, and Sutterella. This shows that reduction of stress relates to the improvement of gut microbiota dysbiosis. In this context, the ability of Chlorella to inhibit stress-induced elevation of glucocorticoids (Hasegawa *et al*., 2000) is relevant, since these hormones are known to reduce GLUT-4 translocation and peripheral glucose utilisation, thus leading to hyperglycaemia, hepatic gluconeogenesis, and insulin resistance (Kuo *et al*., 2015).

The vicious cycle between gut microbiota dysbiosis and chronic low-grade inflammation has been considered to be a relevant factor for the worsening of T2D. Therefore, the improvement of gut microbiota dysbiosis and the control of inflammation induced by Chlorella are of great importance for reducing the complications of T2D, and consequently affecting the stress perception, quality of life, and survival of patients. Our present findings corroborate human and experimental studies in the literature and suggest that changes in microbiota may precede the onset of T2D (DeGruttola *et al*., 2016; Harsch and Konturek, 2018; Upadhyaya and Banerjee, 2015). A comparison of this profile with that of developed countries might support the hypothesis that altered gut microbiota is one of the reasons for the increased incidence of diabetes in the developing world (Upadhyaya and Banerjee, 2015).

In this context, our present findings show the ability of Chlorella to contribute to the restoration of a balanced gut microbiota in patients with T2D. Our findings also strengthen the evidence of the promising use of this alga as an adjuvant in the treatment of diseases related to insulin resistance and in a large number of multifactorial diseases, including inflammatory, metabolic, neoplastic, autoimmune, and neurodegenerative diseases, which have been recently associated with compositional and functional alterations of the gut microbiome (Kho and Lal, 2018; Levy *et al*., 2017). These findings can inform future trials that aim to manipulate the gut microbiome to improve insulin sensitivity and secretion and prevent T2D.

In conclusion, the findings presented here point to the promising use of Chlorella as an adjuvant in the treatment of diseases related to insulin resistance. Moreover, the well-known fact that the role of chronic low-grade systemic inflammation is crucial in the context of infectious diseases, as well as in a wide range of chronic non-infectious diseases (Hunter, 2012), further supports the use of Chlorella as an adjuvant in the treatment of a wide range of pathological conditions.

Increasing evidence that shows that the complex mechanism of action of adaptogens is exerted through interactions with multiple targets is encouraging the development of research fields that favour a multiple target approach. These findings also reveal the clinical benefits associated with a complex biological process that allows for polyvalent beneficial effects against a series of chronic disorders (Panossian, 2017). Altogether, our results emphasise the polyvalent beneficial effects of Chlorella supplementation in chronic low-grade systemic inflammation and add to the relevance of its homeostatic pathophysiological modulation, thus paving the way for a new therapeutic paradigm.

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