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ORIGINAL ARTICLE

# PMA-zeolite can modulate inflammation associated markers in irritable bowel disease – an explorative randomized, double blinded, controlled pilot trial

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## Abstract

**OBJECTIVES:** Preclinical and clinical data suggest, that the microporous mineral with large inner surface and ion exchanger capability PMA-(Panaceo-micro-activation)-zeolite can bind irritating and inflammation associated chime-constituents. We hypothesised whether or not it can ameliorate subclinical inflammation, and investigated the potential in the management of patients with Irritable Bowel syndrome (IBS).

**METHODS:** The trial design was prospective, randomized, controlled, double-blinded, pilot study with 41 patients. They received orally 3 g of the medicinal product PMA-zeolite or microcrystalline cellulose (control) twice a day. At baseline and after three months the symptom load, blood and stool parameters, like high sensitivity C-reactive protein (hsCRP), zonulin, α1-antitrypsin, interleukin IL-10, and changes in the gut microbiome were determined by means of ANOVA, ANCOVA and non parametric statistical analyses.

**RESULTS:** The IBS-associated symptom scores decreased significantly in both groups ($p$=0,001) indicating a strong placebo effect. In the verum but not in the placebo group various inflammation related laboratory parameters decreased. The gross statistical comparison revealed a reduction of α1-antitrypsin significant ($p$=0,037), a lowered inflammation marker hsCRP, paralleled specific microbiome changes (Lactobacillus, Bifidobacteria, and Firmicutes).

**CONCLUSIONS:** The decrease of blood hsCRP and decreased stool α1-antitrypsin suggest that PMA-zeolite possibly can lower inflammation in the gut of IBS patients. The corresponding increase of the immune modulating species Bifidobacteria and Lactobacillus and the reduction of Firmicutes also point at an inflammation ameliorating effect and a possible mucous layer strengthening effect. The protocol of this explorative pilot study is feasible for a sufficiently powered trial on inflammation amelioration in IBS patients.

**Abbreviations:**

| | |
|---|---|
| Al | - Aluminum |
| ANOVA | - ANalysis Of VAriance |
| ANCOVA | - ANalysis of CO-VAriance |
| BASG | - BundesAgentur f. Sicherheit im Gesundheitswesen/ engl.: Fed. Office for Safety in Health Care |
| CFU/g | - Colony-Forming Unit/ gram stool, unit to measure stool bacteria |
| CONSORT | - COnsolidated Standards Of Reporting Trials |
| DNA | - Desoxyribo-Nucleic-Acid |
| ELISA | - Enzyme Linked Immuno Sorbent Assay |
| GSH | - glutathione |
| hsCRP | - high sensitive C-Reactive Protein |
| IBS | - Irritable Bowel Syndrome |
| IL | - Interleukin |
| kD | - Kilo Dalton |
| m0/M0 | - time point: participation start, baseline, (month 0) |
| m3/M3 | - time point: participation end after 3 months (month 3) |
| NGS | - Next Generation Sequencing |
| PMA | - Panaceo-Micro-Activation |
| SD | - Standard Deviation |
| SEM | - Standard Error of the Mean |
| SOD | - Super-Oxide-Dismutase |
| SPP | - species pluralis |
| WMA | - World Medical Association |
| ZC | - Zeolite-Clinoptilolite |

## INTRODUCTION

*Irritable bowel syndrome (IBS)*

Irritable bowel syndrome (IBS) patients report improper function of the small and large intestine. The diagnosis rests on symptoms prevailing for months and the exclusion of other bowel diseases with patho-anatomical changes (Enck *et al.* 2016). The possible mechanisms could involve genetic factors, post-infectious changes, chronic viral and bacterial infections, medication side effects, disturbances in the intestinal microbiome, low-grade mucosal inflammation, immune activation, and altered mucosal permeability (Holtmann *et al.* 2016).

The treatments focus on: dietary restrictions, target global symptoms, psychological status and abdominal pain (Ford *et al.* 2017). IBS can be associated with changes in microbiota and immune status (Raskov *et al.* 2016). Probiotics can reduce visceral hypersensitivity associated with inflammation and psychological stress, improve flatulence and abdominal distension and reduce IBS symptoms scores (Spiller 2008).

In IBS-patients an increased permeability of the colon mucosa is sometimes referred to as "leaky gut" (Gecse *et al.* 2012; Rapin and Wiernsperger 2010) especially in the diarrhoea dominant form (Dunlop *et al.* 2006). Leaky gut can be a danger signal for auto-immune diseases (Mu *et al.* 2017). Chyme constituents - that normally do not cross the gastrointestinal epithelial barrier can overcome the mucosal barrier and trigger symptoms of nutritional intolerance and/ or inflammatory responses. In this context, Mastinu *et al.* (2019) consider the mineral zeolite-clinoptilolite (ZC) an excellent detoxifying, anti-oxidative and anti-inflammatory agent.

*Zeolite-clinoptilolite (ZC)*

Zeolites/clinoptilolites (ZC) are a microporous and crystalline silicate mineral of natural or synthetic origin with characteristic interconnected cavities (Pabalan and Bertetti 2001). The material consists of [AlO4] and [SiO4] tetrahedrons, a negative charge attracts alkali (Na, K, etc.) and/or earth alkali (Mg, Ca, etc.) cations. The loosely attached ions can be easily exchanged for other positively charged also larger molecules (Millini and Bellussi 2017). The large internal surface increases the selective ion-exchange capacity. (Danilczuk *et al.* 2008; Jurkic *et al.* 2013; Thompson and Darwish 2019), and vindicates their use in human medicine (Bacakova *et al.* 2018; Mastinu *et al.* 2019).

ZC can bind and inactivate living bacteria (Prasai *et al.* 2016, 2017; Weiss *et al.* 2010). Also zeolites in dog food impacts enteral microbiota (Sabbioni *et al.* 2016). Disruption of the physiologic symbiotic relationship (eubiosis) between the human host and the microbiota may contribute to IBS (Raskov *et al.* 2016). Preclinical studies revealed positive effects on the microbial intestinal population (Kraljević Pavelić *et al.* 2018). In chicken fed with natural ZC, Wu *et al.* (2013) describe a significant reduction of Escherichia coli ($p$ <0.05) and a significant increase of Lactobacillus acidophilus (Almeida *et al.* 2014). In laying hens the supplementation with a natural ZC caused a significant reduction of Enterobacteriaceae – a pathogen-rich family, while symbiotic bacteria were found un-disturbed (Prasai *et al.* 2017).

In humans, the medicinal product Panaceo Micro Activation Zeolite-Clinoptilolite (PMA-ZC) increases the tolerance towards chemotherapy in patients treated for colorectal cancer (Vitale *et al.* 2020) and can reduce or even eliminate post-therapeutic effects when given during or after aggressive therapies (Eisenwagen and Pavelić 2020). Only natural zeolites with a high silica content are chemically stable in gastric acid (Payra and Dutta 2003). Consequently, not every zeolite variant is a priori safe, for human use, adequate testing and certification is mandatory. For our study we opted for PMA-ZC as study substance, also because it is known that after ingestion no lead is released (Kraljević Pavelić *et al.* 2018).

*Study rational and hypothesis*

In view of the preclinical and clinical findings on the therapeutic potential, we investigated the supplementation of the medicinal product PMA-ZC in IBS patients, and focussed on clinical, patho-physiological and safety endpoints.

## METHODS

*Trial design*

To find possible endpoints we realized an explorative pilot study The study design was prospective, two-arm (PMA-ZC; microcrystalline cellulose), parallel group,

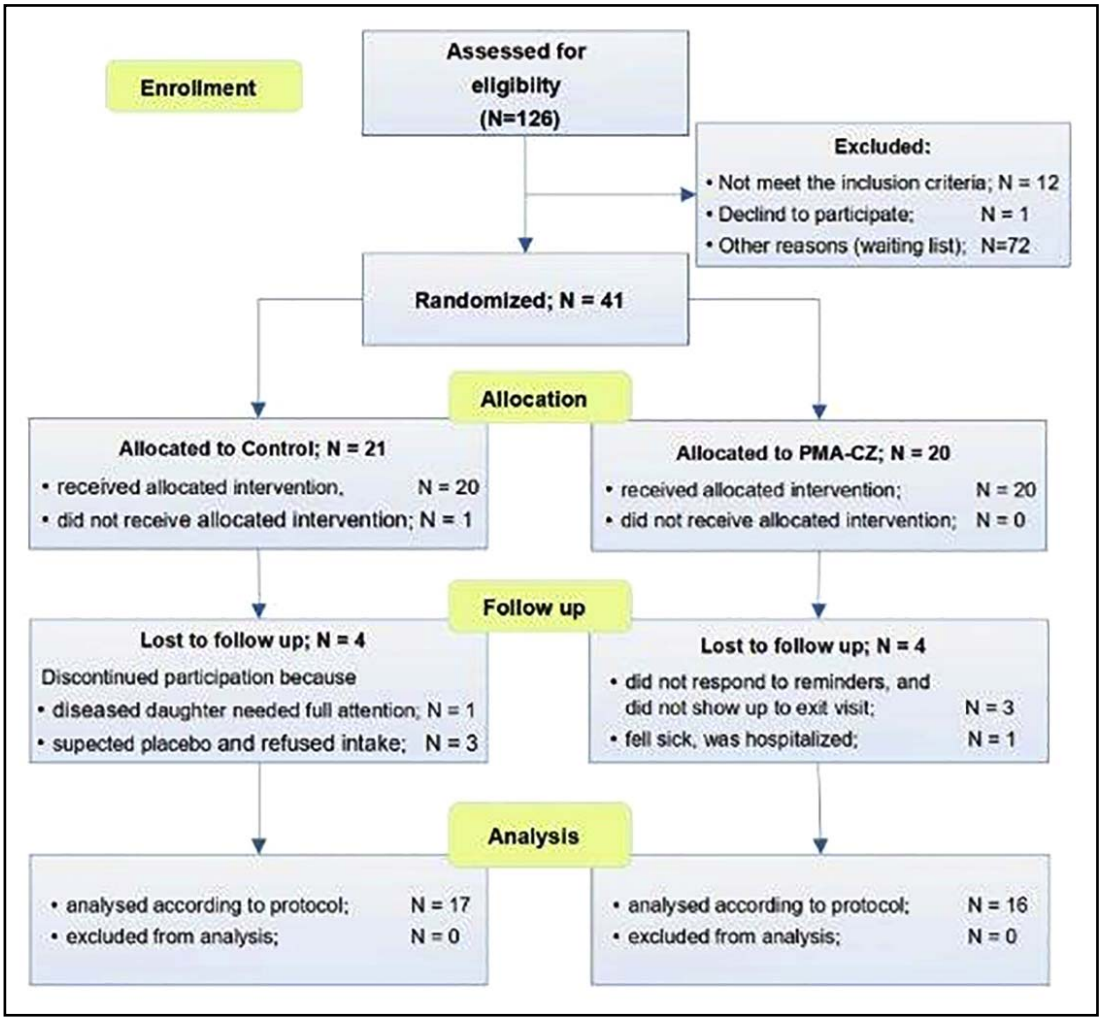


**Fig. 1.** CONSORT diagram, project flow

randomized, double-blinded, controlled clinical trial in an outpatient setting. A medical laboratory was the study centre; offices of four medical doctors were points of patient-enrolment.

The study participants were diagnosed with IBS according to the Rome III criteria (Rome Foundation, USA). This diagnostic standard recruits patients with "discomfort", next generation (Rome IV criteria) focusses on "pain". A validated German translation of the criteria was used. The patients were advised to maintain their lifestyle and medication during the three months intervention phase. At start they provided detailed information about their dietary habits and were instructed to report any changes of their normal eating habits. Furthermore, to minimize changes of the daily diet, the participants agreed in written to abstain from travel activities during the intake phase.

*Ethical aspects and registration*

The trial protocol was approved by the Ethics Committee of the City of Vienna (EK17-171-1017), registered and approved by the national "federal office for safety in health care" (BASG-Reference 10574347) and registered at www.clintrials.gov (identifier NCT03817645).

The protocol complied with the WMA's declaration of Helsinki for Research in Human Subjects 1964 and the amendments since then. Conceivable study related casualties were covered by a patient insurance. The randomization key was kept by a third party and forwarded to the study-team for statistical analyses after the dataset was closed and first descriptive statistics were done.

*Participation*

A total of 41 subjects were recruited according to the following inclusion criteria i) age between 18 and 80, ii) signed informed consent, and iii) diagnosed with IBS, a discomfort/pain frequency "per week or higher" (Rome score of 4-6). Females of childbearing potential delivered a negative pregnancy test before they could participate. Patients with one or more of the following conditions were excluded: known chronic inflammatory diseases, like M. Crohn or Colitis ulcerosa; recent or ongoing cancer therapy, diagnosed food intolerance, neurologic or psychiatric disease, chronic constipation, lose weight medication, alcohol and drug abuse,

recent or ongoing immunosuppressive therapy, recent or actual intake of any zeolite.

The randomization was done 1:1 in blocks of four, using the online randomization tool https://www.sealedenvelope.com/simple-randomiser/v1/lists [accessed: 22 Jan 2021].

For each participant, the study substances were delivered to the study team, blinded, randomized, and numbered, and were packed in carrier bags together with the study specific documents. To prevent deciphering, the recruited patients allocated themselves to the respective study arm by picking their personal bag out of a stack. As no adverse reactions were to be expected, no particular rule for early termination was defined.

There was no follow up of patients withdrawn from the clinical investigation. Early dropouts (within a week from study start) were replaced by the next subject on the waiting list. Later dropouts were not replaced and therefore reduced the sample size for data analysis.

The sampling of blood and stool was integrated in the daily routine of the study centre. After the baseline blood and stool samples were taken, each patient picked the personal bag with the study substance and documents.

Participants taking any medications were instructed to take it at least 2 hours separated from the intake of the study substance. The intake of the study substance (taken twice a day at a single dose of 3 g), concomitant medications (e.g. laxatives), number of bowel movements, and stool consistency (Bristol scale) were documented in a diary.

To enhance the participants compliance a telephone hotline was installed. A study-team-member called each participant at least twice during the intake phase for feedback and encouragement, and checked whether the instructions (diary-documentation, etc.) were clear.

During the exit visit blood samples, stool samples (not older than 24 hours) and their completed diary and questionnaires were collected. An incomplete participation was defined as <80% documented days in the diary.

*Study substance*

The verum substance was the medicinal product PMA-ZC, manufactured by Panaceo International GmbH (Goedersdorf, Austria). The control substance was 3 grams of commercial microcrystalline cellulose, as used before (Lamprecht *et al.* 2015; Vitale *et al.* 2020). As common ingredient of pharmaceutical preparations, it is considered safe (Burdock 2007), taken at larger doses it may modulate specific microbiome species (Prasai *et al.* 2017) but the amount given here is far below the typical daily intake during a normal diet. Both study substances were labelled indicating the intended use in a clinical investigation. The dose for a single intake was packed in "ready to use" paper-sachets. In addition to the written instructions, the participants could see on a video how to stir up the sachet-content in a glass of water and drink it swiftly.

*Endpoints*

The following endpoints were documented at participation start (baseline; m0) and after three months (m3) of intake:

- Symptoms: Rome III diagnostic tool for IBS
- Symptoms: Rome III constipation module (6 items)
  1. strong presses during bowel movements
  2. lumpy or hard stools
  3. feeling of incomplete emptying
  4. Feeling of anorectal obstruction / blockage)
  5. manual maneuvers to facilitate defecation
  6. less than 3 empties per week
- Blood laboratory and stool laboratory to document signs of Intestinal wall integrity, acute and chronic inflammation, Aluminium leakage, etc.
- Exploratory microbiome analyses.

The tests and equipment used for the laboratory parameters were maintained and calibrated according to the standard surveillance rules for medical laboratories (EC-standards). A full red and white blood cell count was carried out at participation start to roughly check the health status. The other blood and stool parameters served as endpoints and were determined at baseline and study exit.

The stool samples for the microbiome analyses by rDNA sequencing were frozen and analysed collectively to minimize possible inter-batch variance. To investigate a possible impact of PMA-ZC on the microbiome, various bacteria from stool samples were determined by NGS (Next Generation Sequencing) of the 16S rDNA (hypervariable V3 and V4 regions; Illumina MiSeq instrument. Takara Bio Europe SAS, France). The microbiome data are expressed as colony forming unit per gram stool (CFU/g) or as ratio between specific species or phyla.

The genomic bacterial DNA was extracted automatically from the stool samples on a MagNA Pure 96 system (F. Hoffmann-La Roche Ltd, Germany) using the MagNA Pure 96 DNA and Viral NA Small Volume Kit. The extracted genomic DNA was amplified, quantified flourimetrically, normalized and provided with indices (Nextera XT DNA Library Preparation Kit Illumina, Eindhoven, Netherlands). The amplicons were pooled in preparation for the actual sequencing and with MagSi-NGSPREP Plus - Magnetic Beads for NGS (MagnaMedics GmbH, Aachen, Germany) and the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, Darmstadt , Germany) checked for their quality and size. The Amplikon Library was then combined with a PhiX control library (at 20%). The library pool was clustered to a density of approximately 500–750 K/mm². The prepared libraries were sequenced in 300PE MiSeq runs. The resulting sequencing data matched the specifications with Q30average> 75%.

Image analysis, base calling, data quality assessment and demultiplexing were carried out with the MiSeq instrument. For the bioinformatic evaluation the software package QIIME (V.1.8.0) was used, the sequences were quality trimmed and paired end aligned. The taxonomic classification of the microbial communities was performed by assigning the resulting sequences to operational taxonomic units (OTUs) using the USEARCH6.1 algorithm with a 97% threshold for pairwise identity and classifying them taxonomically (RDP classifier), both based on the Greengenes database as well as the HITdb (Human Intestinal Tract database).

*Data processing and Biometry*

The data – at baseline and after three months - were transferred from the questionnaires to a spreadsheet, or downloaded from the laboratory's electronic data storage system, and verified against the original laboratory printouts. Missing data were subject of queries. The completed and verified data set was sealed, a copy was used for statistical analyses.

The statistical analysis was carried out with the IBM program SPSS Statistics V.26, using descriptive and analytical methods. Depending on the data distribution parametric methods for analysis of variance (ANOVA) or Covariance (ACOVA), alternatively non-parametric methods like Mann-Whitney U or Wilcoxon were used. In case of multiple testing the *p*-value correction was according to Bonferoni. A p-value of < 0,05 was considered significant. The specific statistical method employed is given in the respective result section.

## RESULTS

*Study subjects*

A CONSORT diagram shows the case numbers from enrolment to analyses (Figure 1).

Table 1 summarizes the participants' age, gender and group-allocation. Most of the participants preferred mixed diet, six females were vegetarian, no participant was vegan, three participants were smokers. None of the 41 participants showed any abnormalities in the red and white blood cell count. None of the participants reported any change in lifestyle, medication, and/or eating habits during the intake phase.

A total of 33 subjects completed the three-month supplementation and daily diary documentation according to the protocol. All dropouts were females. Three of them suspected to have "picked a placebo", which they considered "inacceptable", one participant had a case of serious illness in the family, one participant was hospitalized and seized the regular intake, three participants gave no reason. There was no early termination due to an adverse event or adverse health reaction.

A few single parameters were lost, e.g. stool sampled by the participants were delivered "dry" and not in the stabilizer liquid as required. The dropout rate of single parameters was 4,59% from 675 single laboratory-parameters expected.

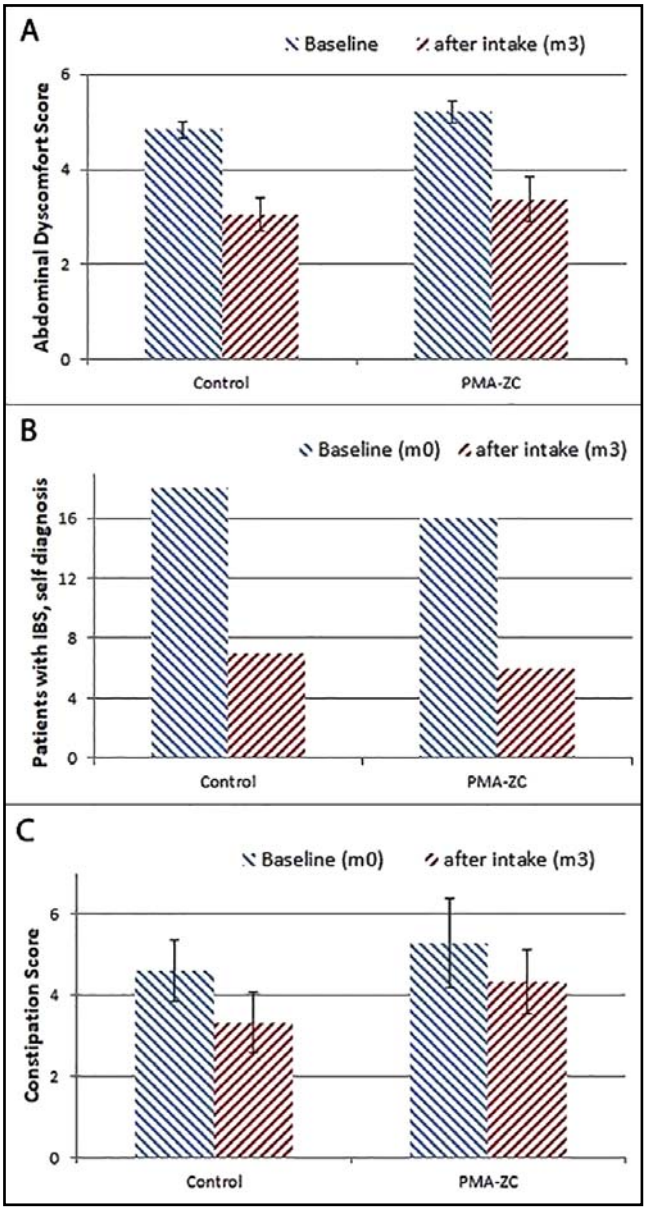


**Fig. 2.** Subjective symptoms at baseline (m0) and after 3 months (m3)
A) abdominal discomfort score ± SEM, B) IBS self diagnosis, C) constipation score ± SEM. The symptom scores decreased at similar rates in both groups, suggesting a strong placebo effect. The change of constipation over time is not significantly different (ANCOVA, P= 0,588). The data do not indicate a constipating effect due to PMA-zeolite but indicate that the participants benefited from study-participation itself.

*Subjective Symptoms*

The score for "abdominal discomfort" decreased from m0 to m3 in both groups almost identically (Mann-Whitney U; $p = 0.932$, Figure 2)

After three months (m3) the criteria for IBS diagnosis (association of abdominal discomfort with stool frequency and stool consistency) improved in both groups at equal rates (Figure 2).

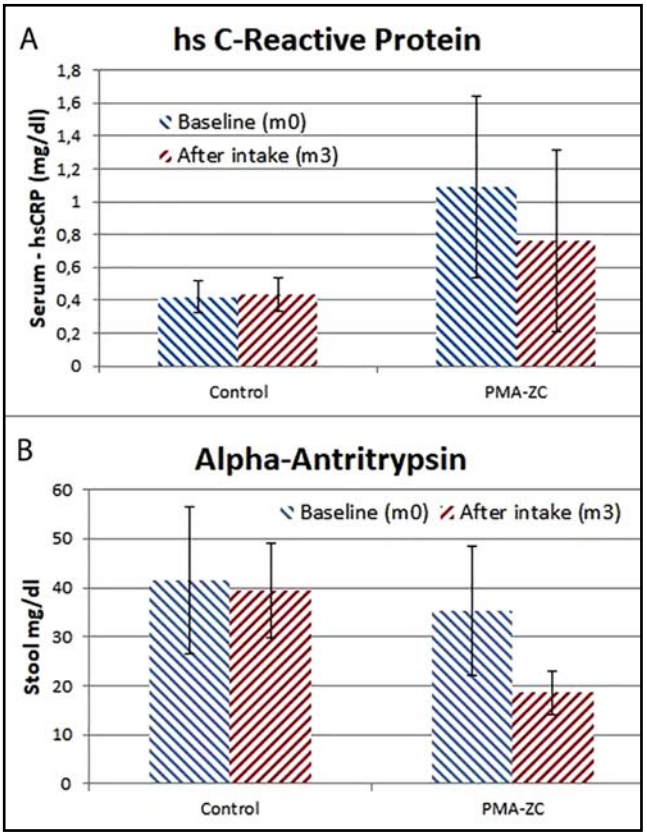


**Fig. 3.** Inflammation related laboratory parameters, baseline (BL, m0) and after the three month ingestion (m3); A) high sensitive C-reactive protein (hsCRP), B) α1-antitrypsin. While in the control group the levels remain roughly the same, the regular intake over three months (m3) was associated with a substantial reduction of the average hsCRP levels and α1-antitrypsin levels in the PMA-ZC- group suggesting some improvement of the inflammatory status and the gut mucosa barrier function.

A possible constipating effect of PMA-zeolite documented as Rome III module "C3 Functional Constipation" was not confirmed. The constipation scores decreased in both groups at similar rates (Figure 2).

*Laboratory parameters*

Table 2 shows the results of the laboratory endpoint analyses. At baseline the ammonium serum levels of all participants were low. During the supplementation phase the values increased in both groups, more pronounced in the control group. The benefit in the PMA-ZC-group was not statistically significant (Table 2, item 5).

Apart from a few outliers most of the obtained histamine-values were "0" (or closeby), which prevented a substantial analyses and group comparison. The DAO activity in the serum was determined in parallel to the stool histamine and was similar in both groups. The activity increased during the observation time in both groups, the difference between the groups was not statistically significant (Table 2, item 6).

Aluminium (Al) in whole blood was low at baseline and even lower after three months in both groups (Table 2, item 9).

*Inflammation Marker*

While the hsCRP values in the placebo group remained almost unchanged, the values in the PMA-ZC-group started high and dropped during the intake phase (Figure 3). Due to the larger SEM in the PMA-ZC-group, the difference to the controls was not statistically significant (Table 2, item 7).

While the α1-Antitrypsin values remained at the same level in the placebo group, a substantial reduction in the PMA-ZC-group was observed (Table 2, item 3). In the PMA-ZC-group, despite a slightly lower value at baseline, the mean value decreased sharply after three months (m3) treatment (Figure 3).

Calprotectin is a marker for acute inflammation in the intestinal tract. In both groups, the calprotectin content decreased during the intake phase. The difference between the groups was not significant (Table 2, item 4).

*Other laboratory findings*

The analysis of IL-10 values was not possible because only four measures at time point m0 were above the tests' detection threshold (5 pg/ml; data not shown).

After three months of intake, stool-zonulin decreased, indicating an improvement of the mucosal barrier function. The improvement is more pronounced in the PMA-ZC-group, whereas the group difference is not statistically significant (Table 2, item 2).

*Microbiome*

Table 3 provides the details of the microbiome analyses: biodiversity, prevalence of individual species, and ratio between species before and after the intake phase. Although not statistically significant, improvements were observed with specific microbiome inflammation associated species like Lactobacillus, Bifidobacteria, and Firmicutes. (Table 3).

## DISCUSSION

The synopsis of the laboratory parameter shifts (reduced blood hsCRP and reduced stool- α1-antitrypsin) suggests a mild anti-inflammatory effect. The observed increase of Bifidobacteria and Lactobacillus species in the PMA-ZC-group further supports this finding. Wu *et al.* (2013) described a ZC-supplementation associated reduction of Escherichia coli ($p$ <0.05) and a significant increase of Lactobacillus acidophilus. As cations from ZC can serve as cofactors for anti-oxidative enzymes like SOD (superoxide dismutase) and GSH (glutathione) (Ivkovic *et al.* 2004), anti-oxidative activities are another possible explanation for the proposed anti-inflammatory effect (Montinaro *et al.* 2013).

**Tab. 1.** Recruited IBS Participants; age, gender and group allocation. The groups were similar in age and gender.

| Gender | Age (mean +- SD) | Control (N) | PMA-ZC (N) | Total (N) |
|---|---|---|---|---|
| Male | 56.7 ± 22.5 | 6 | 5 | 11 |
| Female | 51.6 ± 19.5 | 15 | 15 | 30 |
| Total | 52.9 ± 28.0 | 21 | 20 | 41 |

Although males and females were diagnosed with IBS at equal rates, more females than males were recruited, but the randomization and the group allocation distributed males and females equally to both groups.

The analysis of subjective symptoms revealed a strong beneficial effect, attributable to the study participation itself. Figure 2 reveals the number of participants, who had lower symptom-scores and – after the participation – had lost the status IBS-patient in both groups. Also, the reduced "abdominal discomfort" and the decrease of the score for constipation – a suspected side effect of PMA-ZC supplementation was observed in both groups at comparable rates (Figure 2). The data did not confirm a constipating side effect, associated with the regular intake of PMA-ZC (Figure 2).

PMA-ZC contains covalently bound Aluminium, which constitutes a theoretical safety concern. i.e. leakage and absorption of Al. The observation, that the intake of PMA-ZC actually lowered the Aluminium levels (Table 2, item 9) is consistent with findings of Kraljevic-Pavelic *et al.* (2017) and Lamprecht *et al.* (2015), who showed that PMA-ZC actually reduces the Aluminium-burden in $Al_4Cl$ intoxicated rats or humans, respectively.

Since ZC is described as effective in the absorption of ammonium (Demir *et al.* 2002; Sprynskyy *et al.* 2005), it was unexpected that ammonium has increased in both groups rather than decreased in the PMA-ZC-group (Table 2, item 5).

As the recruited participants were not screened for a potential histamine intolerance syndrome, it is no surprise that the investigation of histamine in stool and the histamine metabolizing DAO did not reveal resilient results.

A frequently discussed etiologic factor for IBS is low grade silent inflammation associated with food intolerance, immunological phenomena, subclinical infection, or other causes. The marker for acute intestinal inflammation – calprotectin in stool – decreased in both groups, even more in the control group (Table 2, item 4) indicating an unspecific finding in a rather healthy study population.

Lamprecht *et al.* (2015) described that three months of PMA-ZC-supplementation exerts beneficial effects on intestinal wall integrity, accompanied by mild

**Tab. 2.** Stool and blood laboratory parameters, at baseline (m0) and after intake (m3), statistical analyses. After Bonferoni *p*-value correction stool *p* values under 0,0125 are statistically significant.

| Group | | | Control Mean +- SEM (N) | | PMA-ZC Mean +- SEM (N) | | |
|---|---|---|---|---|---|---|---|
| Item | Parameter | Unit | Baseline M0 | After Intake M3 | Baseline M0 | After Intake M3 | ANCOVA P= |
| Stool | | | | | | | |
| 1 | Histamin | ng/ml | 2938±1494.52 (18) | 16841±14904.80 (16) | 1560±591.32 (19) | 3209±1808.31 (15) | .358 |
| 2 | Zonulin | ng/ml | 77.13±13.85 (21) | 64.29±9.91 (17) | 73.33±12.28 (20) | 52.96±8.87 (16) | .565 |
| 3 | α1-antitrypsin | mg/dl | 43.80±12.85 (20) | 37.63±9.30 (17) | 36.98±9.97 (20) | 18.60±4.43 (15) | .037 |
| 4 | Calprotectin | mg/l | 72.58±17.52 (20) | 44.58±12.28 (17) | 37.55±8.70 (20) | 30.77+-8.10 (15) | .752 |
| Blood | | | | | | | |
| 5 | Ammonium | µmol/l | 72.18±8.00 (21) | 101.10±10.83 (16) | 67.05±8.92 (20) | 89.75±10.55 (16) | .743 |
| 6 | Di-Amino-Oxidase | U/ml | 20.25±2.70 (21) | 19.04±3.23 (16) | 15.00±1.15 (20) | 16.90±2.41 (16) | .510 |
| 7 | hsCRP | mg/dl | 0.59±0.15 (21) | 0.43±0.10 (16) | 1.17±0.51 (20) | 0.76±0.55 (16) | .748 |
| 8 | Il-10. Inter-leukin 10 | pg/ml | 5.41±0.41 (21) | 5.97± 0.97 (15) | 5.00±0.00 (20) | 8.25±3.05 (16) | .328 |
| 9 | Aluminium | µg/l | 4.61±0.17 (21) | 4.11±0.07 (16) | 5.02±0.62 (19) | 4.62±0.26 (16) | .065 |

**Tab. 3.** Microbiom species and phyla, group comparison
Mean and standard deviation (SD); group analyses at baseline (m0) ANOVA, and after three months (m3) by ANCOVA. For better legibility of the large numbers are expressed as exponents of "10"; example: 6,79E+09 = 6,79 x 109 = 6 790 000 000

| Pos | Bact. Species/Family | Group | Baseline (m0) | | | after (m3) | | P/V |
|---|---|---|---|---|---|---|---|---|
| | | | Mean | SD | ANOVA. p= | Diff m0/3 | N | ANCOVA. p= |
| 1 | Bifido-Bact. total | Control | 6.79E+09 | 1.00E+10 | 0.767 | 1.39E+10 | 17 | 0.429 |
| 2 | | PMA | 5.74E+09 | 1.03E+10 | | 7.75E+09 | 16 | |
| 3 | % Bifido.-Adolescens | Control | 59.0 | 33.43 | 0.643 | -1.1 | 13 | 0.144 |
| 4 | | PMA | 64.7 | 24.57 | | 10.2 | 11 | |
| 5 | % Bifido.-Longum | Control | 16.8 | 11.70 | 0.315 | 12.3 | 6 | 0.474 |
| 6 | | PMA | 23.0 | 8.20 | | 1.3 | 6 | |
| 7 | Lactobacillus species | Control | 7.38E+05 | 1.25E+06 | 0.162 | -7.06E+03 | 17 | 0.434 |
| 8 | | PMA | 2.73E+05 | 3.71E+05 | | 7.79E+05 | 16 | |
| 9 | Equol-producing bacteria | Control | 3.43E+09 | 4.10E+09 | 0.171 | 5.27E+09 | 17 | 0.209 |
| 10 | | PMA | 6.60E+09 | 8.32E+09 | | -8.98E+08 | 16 | |
| 11 | Bacteriodetes | Control | 3.15E+11 | 1.75E+11 | 0.166 | -6.89E+10 | 17 | 0.107 |
| 12 | | PMA | 2.39E+11 | 1.27E+11 | | 3.99E+10 | 16 | |
| 13 | % Bacteriodet.Uniform. | Control | 29.4 | 18.80 | 0.451 | -3.5 | 17 | 0.941 |
| 14 | | PMA | 25.3 | 11.41 | | -0.8 | 16 | |
| 15 | % Bacteriodet. Ovat. | Control | 13.4 | 8.60 | 0.588 | -2.5 | 13 | 0.206 |
| 16 | | PMA | 11.9 | 5.32 | | 0.6 | 13 | |
| 17 | Prevotella | Control | 4.67E+10 | 9.53E+10 | 0.714 | -4.68E+09 | 17 | 0.156 |
| 18 | | PMA | 6.24E+10 | 1.44E+11 | | -4.49E+10 | 16 | |
| 19 | Eubacterium Rectale | Control | 9.06E+09 | 5.20E+09 | 0.024 | 6.37E+09 | 17 | 0.451 |
| 20 | | PMA | 5.71E+09 | 2.23E+09 | | 5.47E+09 | 16 | |
| 21 | Eubacterium Hallii | Control | 4.29E+09 | 3.01E+09 | 0.714 | 5.71E+08 | 17 | 0.239 |
| 22 | | PMA | 4.68E+09 | 3.00E+09 | | 1.63E+09 | 16 | |
| 23 | Roseburia Spp | Control | 4.24E+10 | 4.18E+10 | 0.137 | 4.91E+09 | 17 | 0.604 |
| 24 | | PMA | 2.50E+10 | 1.91E+10 | | 2.05E+10 | 16 | |
| 25 | Ruminococcus spp | Control | 2.99E+10 | 2.36E+10 | 0.620 | 9.60E+09 | 17 | 0.880 |
| 26 | | PMA | 3.42E+10 | 2.62E+10 | | 8.24E+09 | 16 | |
| 27 | Butyrivibrio spp | Control | 8.62E+09 | 6.94E+09 | 0.375 | 7.45E+09 | 17 | 0.807 |
| 28 | | PMA | 6.42E+09 | 7.14E+09 | | 1.25E+10 | 16 | |
| 29 | Clostridium Butyricum | Control | 1.29E+10 | 2.69E+09 | 0.827 | 2.53E+09 | 17 | 0.362 |
| 30 | | PMA | 1.32E+10 | 4.20E+09 | | 4.59E+09 | 16 | |
| 31 | Coprococcus | Control | 2.15E+10 | 2.29E+10 | 0.881 | 1.29E+10 | 17 | 0.518 |
| 32 | | PMA | 2.04E+10 | 2.17E+10 | | 7.92E+09 | 16 | |
| 33 | Butyrat producer Total | Control | 1.81E+11 | 7.90E+10 | 0.926 | 3.28E+10 | 17 | 0.579 |
| 34 | | PMA | 1.79E+11 | 7.13E+10 | | 5.15E+10 | 16 | |
| 35 | Clostridia total | Control | 3.28E+09 | 2.71E+09 | 0.286 | 2.17E+09 | 17 | 0.907 |
| 36 | | PMA | 4.62E+09 | 4.27E+09 | | 2.14E+09 | 16 | |
| 37 | Clostridium histolyticum | Control | 6.55E+08 | 4.51E+08 | 0.608 | 4.39E+07 | 17 | 0.666 |
| 38 | | PMA | 5.64E+08 | 5.64E+08 | | 2.14E+08 | 16 | |
| 39 | Christensenellacae | Control | 1.84E+09 | 3.03E+09 | 0.242 | -6.36E+08 | 17 | 0.577 |
| 40 | | PMA | 4.84E+09 | 9.89E+09 | | 4.05E+09 | 16 | |

| Pos | Bact. Species/Family | Group | Baseline (m0) | | | after (m3) | | P/V |
|---|---|---|---|---|---|---|---|---|
| | | | Mean | SD | ANOVA. p= | Diff m0/3 | N | ANCOVA. p= |
| 41 | Fusobact. Spp | Control | 1.56E+07 | 5.54E+07 | 0.794 | -7.15E+06 | 17 | 0.380 |
| 42 | | PMA | 2.08E+07 | 5.71E+07 | | -1.60E+07 | 16 | |
| 43 | Fecalibacteria Prausnitzii | Control | 7.60E+10 | 5.73E+10 | 0.640 | -1.50E+08 | 17 | 0.726 |
| 44 | | PMA | 9.16E+10 | 5.19E+10 | | -3.07E+09 | 16 | |
| 45 | Akkermansia Muciniphilis | Control | 8.64E+09 | 1.79E+10 | 0.088 | -3.48E+09 | 17 | 0.412 |
| 46 | | PMA | 2.11E+10 | 2.26E+10 | | -1.24E+10 | 16 | |
| 47 | Haemophilus | Control | 2.78E+09 | 6.61E+09 | 0.275 | -9.77E+08 | 17 | 0.804 |
| 48 | | PMA | 8.95E+08 | 1.60E+09 | | 1.19E+09 | 16 | |
| 49 | Acinetobacteria | Control | 1.00E+06 | 0.00E+00 | 0.310 | 1.94E+08 | 17 | 0.359 |
| 50 | | PMA | 2.50E+09 | 1.00E+10 | | -2.42E+09 | 16 | |
| 51 | Proteus Species | Control | 2.12E+04 | 4.61E+04 | 0.340 | 4.93E+06 | 17 | 0.325 |
| 52 | | PMA | 1.00E+04 | 0.00E+00 | | 0.00E+00 | 16 | |
| 53 | Enterobacter Spec. | Control | 1.80E+06 | 5.27E+06 | 0.333 | 2.92E+06 | 17 | 0.402 |
| 54 | | PMA | 1.38E+07 | 4.99E+07 | | -1.25E+07 | 16 | |
| 55 | Sulfatreducing Bact. | Control | 8.69E+08 | 8.74E+08 | 0.074 | 1.60E+09 | 17 | 0.304 |
| 56 | | PMA | 1.90E+09 | 2.11E+09 | | 6.20E+08 | 16 | |
| 57 | Oxalobact. Formig. | Control | 2.88E+08 | 4.52E+08 | 0.778 | -2.21E+07 | 17 | 0.245 |
| 58 | | PMA | 3.35E+08 | 4.97E+08 | | -1.67E+08 | 16 | |
| 59 | Escherichia Coli | Control | 7.30E+07 | 8.78E+07 | 0.077 | 6.44E+06 | 17 | 0.914 |
| 60 | | PMA | 2.62E+07 | 5.37E+07 | | 3.81E+07 | 16 | |
| 61 | Enterococc. Spec. | Control | 7.70E+06 | 2.43E+07 | 0.469 | -4.70E+06 | 17 | 0.297 |
| 62 | | PMA | 2.91E+06 | 9.94E+06 | | 1.38E+07 | 16 | |
| 63 | Lactobacillus species | Control | 7.38E+05 | 1.25E+06 | 0.162 | -7.06E+03 | 17 | 0.434 |
| 64 | | PMA | 2.73E+05 | 3.71E+05 | | 7.79E+05 | 16 | |
| 65 | Candida Albicans | Control | 1.06E+03 | 2.43E+02 | 0.322 | 3.47E+03 | 17 | 0.918 |
| 66 | | PMA | 3.44E+03 | 9.75E+03 | | 4.38E+02 | 16 | |
| 67 | Geotrichum candidum | Control | 6.88E+03 | 2.40E+04 | 0.627 | 5.82E+03 | 17 | 0.753 |
| 68 | | PMA | 1.35E+04 | 4.97E+04 | | -5.44E+03 | 16 | |
| 69 | Methanobrevibacter | Control | 2.22E+07 | 5.31E+07 | 0.494 | 7.56E+07 | 17 | 0.338 |
| 70 | | PMA | 5.72E+07 | 2.01E+08 | | -2.21E+07 | 16 | |
| 71 | E. coli | Control | 1.14E+10 | 2.13E+10 | 0.668 | 7.91E+09 | 16 | 0.686 |
| 72 | | PMA | 1.54E+10 | 3.05E+10 | | 1.40E+10 | 16 | |
| 73 | Histamin producing Bact. | Control | 1.01E+09 | 2.96E+09 | 0.186 | -9.47E+08 | 17 | 0.842 |
| 74 | | PMA | 1.00E+07 | 2.14E+07 | | 7.64E+07 | 16 | |

anti-inflammatory effects. Zonulin is a reliable stool biomarker for intestinal permeability analysis in clinical performance (Fasano 2001; Fasano *et al.* 2000). The zonulin levels of our participants decreased more pronounced in the PMA-group, however the benefit from the verum intake was not statistically different from controls (Table 2, item 2). Substantial effects were documented with α1-antitrypsin (Table 2, item 3). The marker represents many serine protease inhibitors and protects tissues from protease damages during inflammation. With a molecular size of approx. 52 kD, it diffuses passively into the intestinal lumen when the intestinal mucosa integrity is compromised (Hundegger *et al.* 1992). Clinical data show that the ELISA (Enzyme

Linked Immuno- Sorbent Assay) is far more sensitive than the conventional method and that it recognises not only hepatic but also enteral α1-antitrypsin. The IDK® α1-antitrypsin ELISA of Immundiagnostik (64625 Bensheim, Germany) was used, as it offers the combination of two specific antibodies widely excluding the possibility of false negative results and thereby enabling a reliable diagnostics of enteral protein loss.

Because silent gut inflammation is among the discussed aetiologies leading to IBS, the α1-antitrypsin data (Table 2, item 3) indicate a beneficial effect attributable to the PMA-ZC-intake (Figure 3). The lowered α1-antitrypsin level in the PMA-ZC-group corroborates a previous observation (Böhm 2020), which renders it unlikely to be an isolated "positive by chance" result. Furthermore, the consistent serum-hsCRP changes (Table 2, item 7) and the consistent changes with inflammation modulating microbial species like Bifido-Bact. and Lactobacillus species (Table 3, items 1–2 and 7–8) support the view of PMA-ZC as "anti-inflammatory tonic" for the gastro-enteral tract.

IBS patients show a different composition of the intestinal microbiota compared to healthy controls in both, quality and quantity (Layer *et al.* 2020). The antimicrobial protein human beta-defensin-2 is increased in IBS patients (Langhorst *et al.* 2009) corroborating the hypothesis that in IBS patients the mucosal innate immune system is activated against a pro-inflammatory response. Guo *et al.* (2017) described that Bifidobacterium infantis and Lactobacillus acidophilus protected the intestinal barrier against IL-1-beta stimulation by normalizing the protein expression of occludin and claudin-1, and preventing IL-1-beta-induced NF-kappa-B activation in CaCo-2 cells (Guo *et al.* 2017). Randomized, controlled studies describe benefits for the IBS patients, although the effect is small (Barbara *et al.* 2012).

Bifidobacteria are the source of short-chain fatty acids, they lower the pH and retard the growth of pathogenic pathogens, which elicits an anti-inflammatory effect in animal models; E.g. in dogs with idiopathic inflammatory bowel disease - the add on probiotic therapies with Bifidobacterium spp. and Lactobacillus spp. were associated with rapid clinical remission (White *et al.* 2017).

Dysbiotic microbiota may promote intestinal inflammation in IBS-patients; in a subset of patients with IBS with constipation, the amount of Lactobacillus and Bifidobacteria correlated with the inflammation protective cytokine Interleukin-10 (Shukla *et al.* 2018). In this explorative pilot study in the verum group protective lactobacilli and anti-inflammatory bifidobacteria increased (Table 3, items 1-2, 7-8), while Fusobacteria - a marker for inflammatory diseases (Chen *et al.* 2018) - decreased (Table 3, items 41-42). The Microbiome composition and the intestinal mucus are closely associated (Paone and Cani 2020).

A suggested impact of PMA-ZC on the gut permeability in IBS patients via microbiome composition is plausible, also because of the existing knowledge of molecular mechanisms in the pathogenesis of IBS as well as progress related to microbiome-derived compounds, metabolites, neuroendocrine factors, and enzymes as reviewed by Mishima and Ishihara (2020).

## CONCLUSION

The data show that the daily intake is safe in respect to Aluminium leakage from the PMA-ZC.

The symptom documentation reveals a study specific white-coat (placebo) effect. The decrease of the blood parameter hsCRP and the decrease of the stool α1-antitrypsin point toward a PMA-ZC mediated anti-inflammatory effect, which is substantiated by the increase of the immune modulating species Bifidobacterium and Lactobacillus and the reduction of Firmicutes species in the microbiome analyses. The results are interesting for clinicians who are aware of the possible role of an intestinal barrier dysfunction in diseases and of the gastrointestinal barrier as a therapy-target in the future (Camilleri 2019).

It is proposed to assess a possible "inflammation ameliorating effect" in a larger study with patients with pronounced signs of inflammation.

## DECLARATIONS

*Conflict of interest*

The company Panaceo International GmbH sponsored the study by compensating the notary deposited the randomization protocol until the finalization of analyses. Panaceo provided furthermore the ready to use substances of investigation, covered the obligatory insurance for study-patients as well as protocol review fees for the competent national authorities and the expenses for laboratory parameters. SE is an employee of the sponsoring company. SE and the company were not involved in the participant recruitment or care, data generation, analysis and interpretation.

The other authors (VP, BS, CM, WM) declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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*Availability of data and materials*

All data generated or analysed during this study are included in this published article. The raw data are available from the corresponding author on reasonable request.

*Authors' contributions*

VP, conception and design; interpretation of the data; critical MS revision

BS, analysis and interpretation of the laboratory data; drafting of the articles methodology section

SE, technical support with the study substances during study rollout

CM, conception and design; analysis and interpretation of the data; dcritical revision

WM, conception and design; analysis and interpretation of the data; drafting of the article; critical revision

All authors read and approved the final manuscript.

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