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# *Post hoc* analysis of fecal samples from responders and non-responders to *Lactobacillus reuteri DSM* 17938 intervention

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**We compared fecal samples from responders and non-responders to administration of *Lactobacillus reuteri* DSM 17938. Data for this *post hoc* analysis were collected from an RCT assessing the efficacy of *L. reuteri* for the management of acute gastroenteritis. Responders were defined as subjects with diarrhea lasting no longer than 48 h. 44 children (17 responders and 27 non-responders) were analyzed. There were no differences in clinical characteristics and gut colonization between both groups. In the responder group, there were significantly lower levels of five metabolites before beginning of the intervention: lactate, choline, ethanol, creatine, and formate. The fecal calprotectin level did not differ between groups prior to the intervention, but its level was significantly lower after intervention in the responder group. Possibly, the responder group with a "metabolic niche", including lower level of metabolites, especially lactate, that are potential products of *Lactobacillus* genus, would determine the response to probiotic treatment. These findings need to be confirmed, but identification of some differences in the fecal metabolomics and the calprotectin level suggests that further studies are warranted.**

**Key words**: probiotics, diarrhea, responders, metabolome, colonization

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**Trial registration**: ClinicalTrials.gov (NCT02989350).
**Abbreviations**: CFU, colony forming unit; CI, confidence interval; ISAPP, International Scientific Association for Probiotics and Prebiotics; ITT, intention-to-treat analysis; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MD, mean difference; NMR, nuclear magnetic resonance; RCT, randomized control trial

## INTRODUCTION

There is interest in determining differences between responders and non-responders to probiotic interventions (Reid *et al*., 2010). Many factors may contribute to the effects of probiotics, including diet, baseline microbiota, dose, matrix, manufacturing conditions, use of medications (e.g., gut microbiota modifiers, such as antibiotics), and diarrhea etiology/vaccination status. Based on animal and human experiments, some recent studies suggest that probiotic administration (or at least combination of 11 strains used by the investigators) does not consistently change the gut microbiota composition and that individual responses to probiotic administration differ. It was also suggested that probiotic effects or lack of them may differ depending on the indigenous microbiota and gene-expression profiles (Suez *et al*., 2018; Zmora *et al*., 2018).

In line with many current guidelines, use of probiotics with documented efficacy may be considered in the management of acute gastroenteritis (Guarino *et al*., 2014, 2018). A 2016 meta-analysis of three randomized controlled trials (n=256) found that when compared to a placebo or no treatment, the administration of *Lactobacillus reuteri* DSM 17938 *(L. reuteri)* has significantly reduced duration of diarrhea and increased the cure rate on day 1 and day 2 (Urbańska *et al*., 2016). However, Szymański and Szajewska found that *L. reuteri* did not reduce the duration of diarrhea when compared to a placebo as an adjunct to rehydration therapy, but it did reduce the duration of hospitalization. That study was a randomized, double-blind, placebo-controlled trial performed with children who had acute gastroenteritis (Szymański & Szajewska, 2019). In this *post hoc* analysis, we compared the fecal properties of responders and non-responders to the administration of *L. reuteri* in participants of the previous trial.

## MATERIALS AND METHODS

This *post hoc* analysis examined fecal samples from responders and non-responders to a probiotic intervention. Data were collected from a randomized, double-blind, placebo-controlled trial that evaluated the effectiveness of *L. reuteri* for the management of acute gastroenteritis in children. Written informed consent was obtained from parents or legal guardians before enrollment. All research was performed in accordance with relevant guidelines.

Full information about this study has been described in detail elsewhere (Szymański & Szajewska, 2019). In brief, the trial included 100 children younger than 5 years of age with acute diarrhea lasting no more than 5 days. The children received *L. reuteri* at a dose of $2\times10^8$ colony-forming units (CFU)/day or a placebo for 5 consecutive days as a supplement to standard rehydration therapy. Of the 100 randomized children, 91 were included in the intention-to-treat analysis (ITT) (*L. reuteri* n=44, placebo n=47). The duration of diarrhea after inclusion in the study was similar in both groups ($p$=0.6). The groups were also similar with respect to all second-

ary outcome measures with one exception. Compared with the placebo group, patients in the *L. reuteri* group had a shorter duration of hospitalization ($p$=0.048). Adverse events were similar in both groups.

In this *post hoc* analysis, only responders and non-responders in the *L. reuteri* group (n=44) were compared. In accordance with recommendations regarding outcomes in the literature (Karas *et al*., 2015), responders were defined as the subjects with diarrhea lasting no longer than 48 h. The subjects with diarrhea lasting more than 48 h were defined as non-responders. The following fecal analyses were performed: assessment of the colonization of the gastrointestinal tract by *L. reuteri*, metabolomic analysis, and assessment of the calprotectin level. Calprotectin is produced by white blood cells and is used as an indicator of the severity of inflammation of the intestine (Burri & Beglinger, 2014).

**Sample collection**. Fecal samples (at least 1 g) were obtained on day 1 (prior to the intervention) and on day 8 (after the intervention). Stool was collected from all children at the start of the study because it was unpredictable as to who would or would not respond to the intervention. However, only data from responders and non-responders in the group treated with *L. reuteri* are presented here. Two samples were obtained from the majority of participants in the study. However, some samples were unsuitable for evaluation because the amount of material collected was too small, and therefore in the end only 19 samples were taken for metabolomic analysis and 21 for calprotectin analysis.

Samples for the assessment of the gastrointestinal tract colonization by *L. reuteri* were collected immediately after stool output by the child. The samples were obtained using a transport tube with Amies transport medium and then stored at +2 to +8°C. After transfer to the laboratory, samples were plated on the MRS Broth PS60, which is a selective medium for isolation of lactic acid bacteria. The growing strains were then identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The samples used for metabolomic analysis and the determination of fecal calprotectin levels were stored at –20°C until the tests were performed.

**Microorganism identification by the MALDI-TOF MS Biotyper method**. For the MALDI-TOF MS analysis, two to five colonies of actively growing cultures were incubated for 48 h on the MRS agar at 37°C under both, the aerobic and anaerobic conditions, and then suspended in 300 µl of double-distilled water. Next, 900 µl of absolute ethanol were added. The sample was centrifuged two times (13 000×*g*, 3 min), and the sediment was dried at room temperature. Lysates were prepared by adding 50 µl of 70% formic acid to the bacterial pellet, mixing thoroughly, adding 50 µl of acetonitrile, and mixing the sample again.

After centrifugation (13 000×*g*, 2 min), the supernatant was transferred to a fresh tube, and 1 µl of the bacterial protein lysate was applied to a 384 ground steel MALDI target plate (Bruker Daltonics, Bremen, Germany) and air-dried at room temperature. The sample was then overlaid with 1 µl of α-cyano-4-hydroxycinnamic acid matrix solution (HCCA; Bruker Daltonics) and air-dried again. The measurements were performed using a Bruker Daltonics UltrafleXtreme spectrometer. Spectra were recorded in a positive linear mode for a mass range of 2 000 to 20 000 Da (laser frequency 200 Hz; ion source voltage one, 25 kV; ion source voltage two, 23.5 kV; lens voltage, 6.0 kV).

Each spectrum was obtained by averaging 1500 laser shots that were acquired from three spot positions under the control of flexControl software 3.1 (Bruker Daltonics). The spectra were externally calibrated using an *E. coli* DH5-alpha standard (Bruker Daltonics). The calibrant consisted of six ribosomal proteins from *E. coli* with added RNAse A and myoglobin to cover a range of 3 637.8 to 16 952.3 Da. Biotyper 3.1 software (Bruker Daltonics) and a database containing 4613 entries were used for identification. According to the manufacturer, the following score values were used: less than 1.7, identification not reliable; 1.7–2.0, probable genus identification; 2.0–2.3, secure genus identification and probable species identification; and more than 2.3, highly probable species identification (van Belkum *et al*., 2015).

**Fecal metabolomic analysis**. Before performing the analysis, the samples were thawed at room temperature and then transferred to plastic tubes, weighed, and lyophilized. After this process, dry feces were weighed again, and methanol extracts of metabolites were obtained. Next, 59±22 mg of feces were placed in plastic Eppendorf tubes. After adding 1 ml of methanol and two steel balls to crush and homogenize the feces, the samples were shaken in a Thermo Mixer at room temperature for 10 min. Next, the specimens were centrifuged at 12 500 rpm at room temperature for 10 min, and then the supernatant was collected and evaporated using a Speedvac instrument.

The dry mass was kept at –80°C until the analysis was carried out. Nuclear magnetic resonance (NMR) spectroscopy was used to analyze the metabolites in the fecal samples. Prior to the NMR analysis, the samples were dissolved in 600 µL of PBS (0.5 M, pH=7.00, 50% $D_2O$, containing $NaN_3$ and 3 mM TSP), vortexed, and centrifuged (12 000 x rpm, 10 min, 4°C). At the end, 550 µL of supernatant were collected into a 5-mm NMR tube (SP, 5 mm ARMAR Chemicals) for measurement.

All NMR spectra were recorded using a Bruker 600 MHz AVANCE II spectrometer with a zgesgp pulse sequence, a relaxation delay of 3.5 s, a time domain of 65 k, and a spectral width of 15 ppm. Manual phase correction was performed using Topspin 3.2 software (Bruker, GmBH, Germany), and baseline correction was performed using MestReNova 12.0.4 software (Mestrelab Research, S.L., Spain). All NMR spectra were referenced to the TSP resonance (δ=0.000 ppm).

The resonance signals were aligned using the correlation optimized warping algorithm (COW) (Tomasi *et al*., 2004), and icoshift was performed in Matlab (v 8.3, Mathworks Inc.) (Savorani *et al*., 2010). Regions of the spectrum containing residual water and TSP were excluded from calculations. Normalization was performed for all of the spectra using the Probabilistic Quotient Normalization (PQN) method (Dieterle *et al*., 2006). Metabolite resonances were identified using online databases (Biological Magnetic Resonance Data Bank, Human Metabolome Data Base) and assignments published in the literature.

**Fecal calprotectin**. The calprotectin ELISA test (EUROIMMUN) was used to assess fecal calprotectin levels. Measurements were performed according to manufacturer's instructions.

**Statistical analysis**. Nominal variables are presented as $n$ (%), while continuous variables are expressed as the mean or median with the 95% confidence interval (CI) depending on the distribution of the data. Data were tested for normality using the Shapiro-Wilk test. For nominal data, significant differences between groups were detected by conducting a group comparison us-

ing the chi-squared test or Fisher exact test. An independent-samples *t*-test (student's *t*-test) or nonparametric Mann-Whitney *U* test were used as appropriate for continuous data. Additionally, differences in means or medians, depending on the data distribution, were calculated for continuous variables with 95% CIs. All tests were two-tailed, and differences were considered significant at the level of $p<0.05$. All of the analyses were carried out using the statistical software R version 3.4.4 (http://cran.r-project.org).

**Ethics**. The Ethics Committee of the Lower Silesia Medical Chamber approved this study (1&2/PB/2016). The trial was registered at ClinicalTrials.gov (NCT02989350).

## RESULTS

A total of 44 children (median age: 20 months, 95% CI: 12.0; 34.2; 24 boys/20 girls) treated with *L. reuteri* were included in the analysis. Among them, there were 17 responders and 27 non-responders.

### Clinical characteristics

There were no significant differences in any of the clinical characteristics between groups except that there was a higher percentage of females in the responder group than the non-responder group (64.7% *vs.* 33.3%, $p=0.04$) (Table 1). When compared with the non-responder group, there were no significant differences in the responder group in either the duration of diarrhea before enrollment ($p=0.57$) or the severity of diarrhea assessed using the modified Vesikari scale ($p=0.73$).

### Colonization

Colonization of the gastrointestinal tract with *L. reuteri* was confirmed in 14 (31.8%) of the 44 subjects treated with *L. reuteri*. However, there was no significant difference between the responders and non-responders in the percentage of subjects with colonization (8/17 [47%] *versus* 6/27 [22.2%], respectively, $p=0.7$).

### Fecal metabolomic analysis

For the metabolomic analyses, stool samples from only 19 participants were available. Some samples were not provided by caregivers and some of them were not suitable for analysis.

There were no significant differences between the responders (n=8) and non-responders (n=11) with a few exceptions. Levels of five metabolites were significantly lower in the responder group before onset of the intervention: lactate, choline, ethanol, creatine, and formate (Table 2). However, there were no significant differences between the responder and non-responder groups in the levels of any of the metabolites after intervention (Table 3).

### Fecal calprotectin

The fecal calprotectin level did not differ between groups prior to the intervention ($p=0.46$). However, after the intervention, there was a significantly lower level of fecal calprotectin in the responder group (MD 213.70 $\mu g/g$ feces, 95% CI 25.90 to 443.62; $p=0.03$) (Table 4). In the responder group, there was a significant decrease in the calprotectin level after the *L. reuteri* intervention when compared to the pre-intervention level (MD – 708.9 $\mu g/g$ feces, 95% CI – 1767; – 71.3; $p=0.016$).

## DISCUSSION

### Principal findings

The aim of this study was to compare properties of fecal samples from responders and non-responders to administration of *L. reuteri*. Data for this *post hoc* analysis were collected from a randomized, double-blind, placebo-controlled trial that evaluated the effectiveness of *L. reuteri* for the management of acute gastroenteritis in children. There were no significant differences in clinical characteristics between responders and non-responders except that the response was more likely to occur in females. Colonization of the gastrointestinal tract with *L. reuteri* was similar in responders and non-responders. However, colonization was confirmed in less than one-third of subjects treated with *L. reuteri*.

Notably, colonization is not needed for probiotics to exert their beneficial effects, and other mechanisms, such as production of active compounds, may play an important role here (Talarico *et al.*, 1988). There were no significant differences in the fecal metabolite levels between groups with a few exceptions. In the responder group, there were significantly lower levels of five metabolites

**Table 1. Clinical characteristics of non-responders and responders in subjects treated with L. reuteri before onset of the intervention.**

| Characteristics | Non-responders | Responders | $p$[1] |
|---|---|---|---|
| *n* | 27 | 17 | |
| Gender (boys/girls) | 18/9 | 6/11 | 0.04 |
| Age, mo, median (95% CI) | 19.4 (15.5; 26.0) | 23.7 (16.3; 36.3) | 0.37 |
| Vesikari scale, score, mean (95% CI) | 10.3 (9.4; 11.2) | 10.1 (9.0; 11.2) | 0.73 |
| Duration of diarrhea before enrollment, h, mean (95% CI) | 48.2 (37.1; 59.4) | 43.9 (32.4; 55.4) | 0.57 |
| Vomiting, n (%) | 24 (88.9) | 16 (94.1) | >0.99 |
| Fever >38°C, n (%) | 18 (66.7) | 8 (47.1) | 0.22 |
| Bloody stools, n (%) | 4 (14.8) | 0 (0) | 0.15 |
| Rotavirus, n (%) | 11 (40.7) | 7 (41.2) | 0.98 |
| Adenovirus, n (%) | 0 (0) | 0 (0) | >0.99 |

[1]Student's *t*-test, Mann-Whitney U test, Chi-squared test, or Fisher exact test as appropriate

**Table 2. Metabolite levels before onset of the intervention in non-responders and responders in subjects treated with *L. reuteri* (data available for 19 out of 44 subjects).**

| Metabolites | Non-responders[1] | Responders[1] | $p$[2] |
|---|---|---|---|
| *n* | 11 | 8 | |
| Formate | 0.06 (0.04; 0.18) | 0.03 (0.02; 0.13) | 0.028 |
| Unknown | 0.06 (0.03; 0.09) | 0.06 (0.01; 0.12) | 0.92 |
| Xanthine | 0.07 (0.04; 0.09) | 0.09 (0.05; 0.13) | 0.23 |
| Uracil | 0.21 (0.09; 0.34) | 0.29 (0.18; 0.40) | 0.33 |
| Phenyloalanine | 0.56 (0.31; 0.80) | 0.44 (0.24; 0.63) | 0.43 |
| Tyrosine | 0.25 (0.17; 0.33) | 0.24 (0.14; 0.34) | 0.83 |
| Lactate | 3.32 (1.99; 6.95) | 0.61 (0.39; 4.08) | 0.016 |
| Creatine | 0.08 (0.02; 0.14) | 0.01 (0.00; 0.02) | 0.022 |
| Methanol | 1.20 (0.61; 43.96) | 0.58 (0.33; 11.8) | 0.4 |
| Choline | 1.34 (0.79; 1.90) | 0.28 (0.09; 0.47) | 0.002 |
| Trimethyloamine | 0.03 (0.02; 0.49) | 0.08 (0.02; 0.23) | 0.97 |
| Succinate | 0.67 (0.42; 3.78) | 1.31 (0.59; 6.56) | 0.44 |
| 4-aminobutyrate | 0.33 (0.19; 0.69) | 0.42 (0.22; 0.75) | 0.49 |
| Propionate | 0.57 (0.33; 0.80) | 0.89 (0.42; 1.36) | 0.14 |
| Methionine | 0.28 (0.11; 0.45) | 0.31 (0.14; 0.48) | 0.78 |
| Unknown | 0.24 (0.21; 1.77) | 0.22 (0.16; 0.55) | 0.18 |
| Acetate | 5.99 (3.51; 10.64) | 6.88 (3.60; 13.67) | 0.84 |
| Alanine | 2.50 (1.93; 5.10) | 5.6 (2.86; 9.00) | 0.15 |
| Metylomalonate | 0.18 (0.13; 0.60) | 0.11 (0.08; 0.19) | 0.13 |
| Ethanol | 0.21 (0.11; 0.31) | 0.06 (0.03; 0.09) | 0.007 |
| Isobutyrate | 0.88 (0.41; 0.35) | 0.79 (0.48; 1.10) | 0.75 |
| Valine | 1.07 (0.51; 0.64) | 1.02 (0.51; 1.53) | 0.88 |
| Isoleucine | 1.80 (0.85; 2.76) | 1.58 (0.89; 2.27) | 0.72 |
| Leucine | 0.07 (0.24; 0.90) | 0.12 (0.08; 0.24) | 0.18 |
| Butyrate | 0.55 (0.24; 0.87) | 0.84 (0.38; 1.30) | 0.24 |

[1]Values are means (95% CI) or medians (95% CI). [2]Groups were compared with the use of the Mann-Whitney U test or Student's *t*-test for continuous variables

(lactate, choline, ethanol, creatine, and formate) before beginning of the intervention. The fecal calprotectin level did not differ between groups prior to the intervention. However, there was a significantly lower level of fecal calprotectin after the intervention in the responder group. Additionally, responders showed a significant reduction in the fecal calprotectin level after the intervention when compared to the pre-intervention level.

## Strengths and limitations

The strengths and limitations of the original trial were discussed in our earlier publication (Szymański & Szajewska, 2019). In brief, the strengths included the methodology: the study protocol was published prior to the enrollment of the first subject, there was adequate generation of the allocation sequence and allocation concealment, there was adequate blinding, and an intention-to-treat analysis was performed. One of the limitations is that there was no formal confirmation of the identity of the organism or the number of CFUs in the study product. However, in a parallel study (Kołodziej & Szajewska, 2019), both study products were blindly tested using genetic methods, and the active product was confirmed to contain *L. reuteri* DSM 17938.

One limitation of the current responder/non-responder analysis is the *post hoc* design, which precludes robust conclusions. However, it does allow generation of hypotheses for future studies (Curran-Everett & Milgrom, 2013). Additionally, responders and non-responders were not predefined. It may be argued that defining responders as those with diarrhea lasting no longer than 48 h is not optimal. However, according to the literature the proportion of patients who recover by 48 h is one of five outcomes for therapeutic studies (Karas *et al.*, 2015). Another limitation is the small number of subjects available for analysis. However, the sample size was calculated for the primary outcome of the original trial. Thus, caution is needed when interpreting findings of this *post hoc* analysis.

Despite limitations of the *post hoc* analysis, we decided to perform it since there is interest in the question of why some subjects respond to probiotic interventions

**Table 3. Metabolite levels after the intervention in non-responders and responders in subjects treated with *L. reuteri* (data available for 19 out of 44 subjects).**

| Metabolites | Non-responders [1] | Responders[1] | $p$[2] |
|---|---|---|---|
| *n* | 11 | 8 | |
| Formate | 0.04 (0.03; 0.23) | 0.02 (0.02; 0.16) | 0.35 |
| Unknown | 0.06 (0.03; 0.10) | 0.05 (0.03; 0.07) | 0.73 |
| Xanthine | 0.09 (0.06; 0.11) | 0.10 (0.07; 0.13) | 0.44 |
| Uracil | 0.22 (0.16; 0.28) | 0.26 (0.18; 0.34) | 0.35 |
| Phenyloalanine | 0.33 (0.24; 0.42) | 0.34 (0.21; 0.46) | 0.88 |
| Tyrosine | 0.22 (0.15; 0.28) | 0.25 (0.15; 0.36) | 0.51 |
| Lactate | 0.70 (0.52; 2.28) | 0.43 (0.28; 2.18) | 0.21 |
| Creatine | 0.02 (0.01; 0.04) | 0.03(-0.00009; 0.06) | 0.63 |
| Methanol | 5.69 (1.29; 39.06) | 17.79 (4.59; 44.29) | 0.24 |
| Choline | 0.18 (0.12; 0.27) | 0.17 (0.07; 0.46) | 0.84 |
| Trimethyloamine | 0.10 (0.04; 0.15) | 0.05 (0.01; 0.08) | 0.15 |
| Succinate | 0.66 (0.33; 2.42) | 0.54 (0.24; 4.86) | 0.97 |
| 4-aminobutyrate | 0.21 (0.09; 0.47) | 0.05 (0.03; 0.22) | 0.11 |
| Propionate | 1.08 (0.84; 1.99) | 1.27 (0.66; 2.44) | 0.66 |
| Methionine | 0.22 (0.17; 0.27) | 0.23 (0.11; 0.36) | 0.86 |
| Unknown | 0.41 (0.31; 1.22) | 0.45 (0.26; 1.11) | 0.92 |
| Acetate | 10.71 (7.12; 14.29) | 11.81 (7.66; 15.97) | 0.65 |
| Alanine | 3.44 (2.29; 4.59) | 3.34 (2.02; 4.65) | 0.89 |
| Metylomalonate | 0.14 (0.11; 0.32) | 0.14 (0.07; 0.40) | 0.49 |
| Ethanol | 0.05 (0.04; 0.07) | 0.04 (0.03; 0,07) | 0.23 |
| Isobutyrate | 0.60 (0.41; 0.78) | 0.56 (0.29; 0.84) | 0.81 |
| Valine | 0.83 (0.57; 1.10) | 0.80 (0.41; 1.19) | 0.87 |
| Isoleucine | 1.16 (0.81; 1.52) | 1.11 (0.64; 1.57) | 0.82 |
| Leucine | 0.10 (0.08; 0.12) | 0.10 (0.05; 0.16) | 0.86 |
| Butyrate | 1.14 (0.68; 2.08) | 1.13 (0.44; 2.41) | 0.97 |

[1]Values are means (95% CI) or medians (95% CI). [2]Groups were compared with the use of the Mann-Whitney U test or Student's *t*-test for continuous variables

**Table 4. Comparison of the calprotectin level (µg/g feces) between groups prior to the intervention and after the intervention.**

| | Non-responders | Responders | $p$ |
|---|---|---|---|
| *n* | 13 | 8 | |
| Calprotectin before the intervention | 206.2 (114.3; 1397.1) | 762.1 (106.3; 1890.2) | 0.46 |
| Calprotectin after the intervention | 267.0 (166.2; 1026.4) | 53.3 (10.8; 321.0) | 0.03 |

Values are medians (95% CI)

while others do not. In 2010, the International Scientific Association for Probiotics and Prebiotics (ISAPP) addressed this issue (Reid *et al.*, 2010). The ISAPP experts formulated four recommendations. First, the end goal of the study needs to be clearly defined. Second, the study design should "maximize the chance of a positive response by identifying precise parameters and defining the level of response that will be tested." Third, the selection of probiotic strains should be based on solid scientific criteria. Fourth, the study cohort should be selected carefully.

With respect to the fourth recommendation, the experts suggested that studies "use biological or genetic markers when available to stratify the patient population before enrollment and decide at what point the intervention will provide the best outcome (for example, in the acute phase of the disease, during remission, and with or without use of pharmaceutical agents)." Thus, the ISAPP experts indicated the importance of using metabolomics in clinical trials assessing probability of a positive response to treatment with a given probiotic.

In that sense, our study meets the ISAPP recommendations. However, as stated earlier, this post hoc analy-

sis of responders and non-responders is underpowered for final conclusions. Nevertheless, further evaluation is warranted for metabolomics, which is defined as the "systematic identification and quantification of the small molecule metabolic products (the metabolome) of a biological system (cell, tissue, organ, biological fluid, or organism) at a specific point of time (*Metabolomics – Latest Research and News | Nature*, n.d.)."

In the context of previous studies, it allows to think that there is a "metabolic niche" and that lower level of metabolites, especially lactate, that are potential products of the *Lactobacillus* genus would determine the response to probiotic treatment (Shepherd *et al.*, 2018). The metabolomics approach could be used in diagnosis and management of diseases involving the gastrointestinal tract (Dawiskiba *et al.*, 2014; Lin *et al.*, 2016; Zeber-Lubecka *et al.*, 2016), cardiovascular system (Zordoky *et al.*, 2015), and obstetrics (Miranda *et al.*, 2018) (e.g., eclampsia (Kelly *et al.*, 2017), as well as the study of cancer (Armitage & Ciborowski, 2017; Kaushik & DeBerardinis, 2018), neurodegenerative diseases (Botas *et al.*, 2015), diabetes (Merino *et al.*, 2018), and many others. Thus, the application of metabolomics instrumentation is one of the components of the development of personalized medicine (Jacob *et al.*, 2019). Furthermore, metabolomics and genomics are the methods of choice for studying the microbiome interaction and its influence on the human organism (Holmes *et al.*, 2012; Wang *et al.*, 2019). As an example of the great potential of the metabolomics method, the American Heart Association has recently published a position paper describing the wide range of applications of metabolomics in cardiovascular health and disease, and determining directions for further research (Cheng *et al.*, 2017). It is likely that other scientific societies will also address this issue.

## CONCLUSIONS

This is the first study to attempt to characterize responders and non-responders to an *L. reuteri* intervention. While the findings of this *post hoc* analysis need to be confirmed, identification of some differences in the fecal metabolomics and calprotectin levels suggests that further studies are warranted. Identification of likely responders is needed to help guide the selection of subjects for successful therapies involving *L. reuteri* or other probiotics.

### Authors' contributions

H. Szymański initially conceptualized this study. P. Młynarz, B. Qasem, A. Korzeniowska-Kowal, B. Szponar, and M. Kałwak-Baran were responsible for the analyses. H. Szymański was responsible for data collection. All authors were responsible for data analysis and interpretation. H. Szymański and H. Szajewska assumed the main responsibility for writing of the first draft of this manuscript. All authors contributed to and agreed upon the final version.

### Disclosure statement

H. Szymański, P. Młynarz, B. Qasem, A. Korzeniowska-Kowal, B. Szponar, and M. Kałwak-Baran declare no conflicts of interest.

H. Szajewska has served as a speaker for BioGaia, the manufacturer of *L. reuteri* DSM 17938.

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