Beneficial effects of carotenoid-producing cells of *Bacillus indicus* HU16 in a rat model of diet-induced metabolic syndrome

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RESEARCH ARTICLE

Abstract

A well-established rat model of diet-induced metabolic syndrome was used to evaluate the effects of the oral administration of spores or cells of HU16, a carotenoid-producing strain of *Bacillus indicus*. Symptoms of metabolic syndrome were induced in 90-days old, male Sprague-Dawley rats maintained for eight weeks on a high-fat diet, as previously reported. Parallel groups of animals under the same diet regimen also received a daily dose of 1×10^{10} cells or spores of *B. indicus* HU16. Cells of strain HU16 were able to reduce symptoms of metabolic syndrome, plasma markers of inflammation and oxidative markers in plasma and liver to levels similar to those observed in rats under a standard diet. HU16 cells did not affect obesity markers or the accumulation of triglycerides in the liver of treated animals. Denaturing gradient gel electrophoresis analysis showed that the oral administration of HU16 cells did not significantly affect the gut microbiota of high fat-fed rats, suggesting that the observed beneficial effects are not due to a reshaping of the gut microbiota but rather to metabolites produced by HU16 cells.

Keywords: insulin resistance, metabolic inflammation, *Bacillus indicus* HU16

1. Introduction

Diet plays an important role in the aetiology and prevention of the risk factors for the metabolic syndrome (O'Keefe *et al.*, 2008; Salas-Salvadó *et al.*, 2016). Many epidemiological studies have shown a strong inverse association between consumption of fruits and vegetables and the incidence of cardiovascular diseases (Bazzano *et al.*, 2002; Crowe *et al.*, 2011; Mirmiran *et al.*, 2009). In addition, the intake of antioxidant phytochemicals has been associated with a reduction in the risk of type-2 diabetes (Cooper *et al.*, 2012; Dembinska-Kiec *et al.*, 2008; Hamer and Chida, 2007).

It is known that micronutrient antioxidants, such as vitamins and carotenoids, are decreased in both the serum and liver tissue of patients with chronic liver disease and cirrhosis (Leo *et al.*, 1993; Ruhl and Everhart, 2003; Yadav *et al.*, 2002). In addition, obesity is associated with low serum

concentrations of carotenoids (α -carotene and β -carotene) and vitamin E (Gunanti *et al.*, 2014; Neuhouwer *et al.*, 2001; Ruhl and Everhart, 2003). Thus, these micronutrient antioxidant deficiencies may also contribute to the development of greater adiposity and comorbidities, such as insulin resistance and hepatic steatosis. Importantly, carotenoids are as potent in inhibiting lipid peroxidation as vitamin E (Rock *et al.*, 1996).

The potential health-promoting properties of carotenoids have led to a substantial interest in carotenoids as nutritional supplements, especially from natural sources. Carotenoid-producing bacteria have received interest in recent years due to the fact that many species are thought to be probiotics (Hempel *et al.*, 2012; Ma *et al.*, 2010) coupled with their ability to provide a suitable biosource of carotenoids with both C40 and C30 backbones (Perez-Fons *et al.*, 2010) for the food and feed industry. In particular, spore-forming

bacteria of the *Bacillus* genus have been reported to produce carotenoids with improved stability (Duc *et al.*, 2006) and solubility (Sy *et al.*, 2015). Although these bacteria are not photosynthetic organisms, several isolates produce carotenoids (Khaneja *et al.*, 2010), that are essential for the cellular response to oxidative stress conditions (Manzo *et al.*, 2011, 2013). Spore-forming bacteria share the ability to differentiate, producing a dormant spore, a highly resistant cell form able to survive harsh and extreme environmental conditions. Carotenoids are needed for the protection of cells but not of spores from reactive oxygen species (Manzo *et al.*, 2013), however in some species including *Bacillus indicus* HU36, carotenoids have been found associated with both cells and spores (Duc *et al.*, 2006).

We set out to investigate whether carotenoid-producing bacteria were able to counteract symptoms of metabolic syndrome induced in a rat model using a long-term dietary treatment with a high fat diet. To this aim we used *B. indicus* HU16, a previously characterised bacterium isolated from human faeces with a notable capacity to produce a yellow-orange carotenoid (Khaneja *et al.*, 2010).

2. Methods

Bacterial growth, sporulation and spore purification

B. indicus HU16 (Khaneja *et al.*, 2010) was grown in Luria-Bertani medium at 37 °C in aerobic conditions. Cells were collected at the stationary phase of growth after 18 h of culture, washed two times and stored at -20 °C. Sporulation was induced in Difco Sporulation (DS) medium (Difco, Detroit, MI, USA) by the exhaustion method (Cutting and Vander Horn, 1990). After a 48 h incubation at 37 °C, spores were collected, washed four times, purified by using overnight incubation in H₂O at 4 °C to lyse residual sporangial cells (Nicholson and Setlow, 1990). To obtain the total number of cells and spores needed for the whole experimental period, various preparations were pulled together and counted using a cell counting chamber. 1 ml aliquots, each containing 1×10¹⁰ cells or spores suspended in a 10% (v/v) sucrose solution, were immediately frozen and kept at -20 °C until needed for the animal treatment.

Animals and treatments

Male Sprague-Dawley rats (90-days old; Charles River, Italy) were caged singly in a temperature-controlled room (23±1 °C) with a 12 h light/dark cycle (06.30–18.30). Treatment, housing and euthanasia of animals met the guidelines set by the Italian Health Ministry. All experimental procedures involving animals were approved by Comitato Etico-Scientifico per la Sperimentazione Animale of the University Federico II of Naples. Rats were fed either a standard low fat diet (Group 1, n=6) or a high fat diet (n=18), previously reported to induce symptoms of

metabolic syndrome and obesity (Crescenzo *et al.*, 2008). The dietary treatment lasted eight weeks. The composition of the two diets is shown in Table 1.

Rats fed a high fat diet were subdivided into three groups (each composed of 6 rats), receiving each day by oral gavage 1 ml of a 10% sucrose solution alone (Group 2) or containing 1×10¹⁰ cells (Group 3) or 1×10¹⁰ spores (Group 4) of *B. indicus* HU16 (Table 2).

During the treatments, body weight, food and water intake were monitored daily. Faeces were also collected daily and their energy content assessed with a bomb calorimeter, as previously reported (Crescenzo *et al.*, 2010). At the end of the experimental period, the rats were euthanised by decapitation. Portal blood, systemic blood, caecum and

Table 1. Composition of experimental diets.

	Low fat	High fat
Standard chow ¹ , g	49.98	49.98
Sunflower oil, g	3.25	3.25
Casein, g	8.00	13.49
Butter, g	–	19.49
Corn starch, g	36.48	11.49
AIN Mineral mix ² , g	0.32	0.32
AIN Vitamin mix ² , g	0.13	0.13
Choline, g	0.09	0.09
Methionine, g	0.64	0.64
Total weight, g	100	100
Gross energy density, kJ/g	18.9	22.6
Metabolisable energy density ³ , kJ/g	13.6	18.0
Macronutrient composition, %		
Fat	13	51
Carbohydrate	66	28
Protein	21	21

¹ Commercial non-purified diet (4 RF21, Mucedola, Italy) containing (g/kg): protein 185, lipid 30, fibre 60; metabolisable energy 11.20 kJ/g.

² American Institute of Nutrition, 1993.

³ Estimated by computation using values (kJ/g) for energy content as follows: protein 16.736, lipid 37.656 and carbohydrate 16.736.

Table 2. Treatments layout.

	Group 1	Group 2	Group 3	Group 4
Diet	low fat	high fat	high fat	high fat
10% sucrose	0.5 ml	0.5 ml	–	–
10% sucrose + cells	–	–	0.5 ml	–
10% sucrose + spores	–	–	–	0.5 ml

liver were collected, and the carcasses were used for body composition determination.

Insulin tolerance test, glucose tolerance test, plasma metabolites

Insulin tolerance tests (ITT) were carried out after 4 and 8 weeks of treatment. Rats were fasted for 6 h from 09:00 AM. Basal, post-absorptive blood samples were obtained from a small tail clip and placed in EDTA-coated tubes and then insulin (2.5 IU/kg body weight) was injected by the intra-peritoneal route. Blood samples were collected after 20, 40, 60, 90 and 120 min and placed in EDTA-coated tubes. The blood samples were centrifuged at $1,400\times g$ for 8 min at 4 °C. Plasma glucose concentration was measured by colorimetric enzymatic method (Pokler Italia, Genova, Italy).

Glucose tolerance test (GTT) was carried out after 8 weeks of treatment. To this end, rats were fasted for 6 h from 09:00 AM. Basal, postabsorptive blood sample was obtained from a small tail clip and placed in EDTA-coated tubes and then glucose (2 g/kg body weight) was injected intraperitoneally. Blood samples were collected after 20, 40, 60, 90 and 120 min and placed in EDTA-coated tubes. The blood samples were centrifuged at $1,400\times g$ for 8 min at 4 °C. Plasma glucose concentration was measured by colorimetric enzymatic method (Pokler Italia, Genova, Italy), while plasma insulin concentration was measured using an ELISA kit (Mercodia AB, Uppsala, Sweden) in a single assay to remove inter-assay variations.

Plasma concentrations of alanine aminotransferase, triglycerides, cholesterol and non-esterified fatty acids (NEFA) were measured using a colorimetric enzymatic method using commercial kits (SGM Italia, Rome, Italy). Lipid peroxidation was determined according to Fernandes *et al.* (2006), by measurement of thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid assay.

Plasma lipopolysaccharide (LPS) determinations were performed by a *Limulus amaebocyte lysate* (LAL) kit (Lonza, Basel, Switzerland) using a LAL extract according to the manufacturer's instruction. Briefly, samples were mixed with the LAL reagent and chromogenic substrate reagent for 16 min and absorbance readings were performed on a plate reader at 405 nm. Tumour necrosis factor (TNF)- α concentrations in plasma samples were determined using a rat-specific enzyme linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction.

Body composition and energy balance

After livers removal, guts were cleaned of undigested food and the carcasses were then autoclaved. After dilution in distilled water and subsequent homogenisation of

the carcasses with a Polytron homogeniser (Kinematica, Luzern, Switzerland), duplicate samples of the homogenised carcasses were analysed for energy content using a bomb calorimeter. To take into account the energy content of liver, tissue samples were dried and the energy content was then measured with the bomb calorimeter. Total body water content was determined by drying carcass samples in an oven at 70 °C for 48 h. Total body lipids were measured by the Folch extraction method (Folch *et al.*, 1957). Energy balance measurements were conducted by the comparative carcass technique over the experimental period, as detailed previously (Crescenzo *et al.*, 2010).

Liver composition, hepatic and mitochondrial oxidative stress

Liver triglycerides and TBARS were measured as described above for plasma samples. Isolation of liver mitochondria was carried out as previously reported (Crescenzo *et al.*, 2014). Lipid peroxidation was determined in isolated mitochondria by using the same procedure used for plasma samples.

PCR-DGGE analysis

Bacterial 16S rDNA fragments were amplified using nested PCR. For the primary amplification, PCR was conducted with the primers 27F and 1492R (Chong *et al.*, 2009). The PCR mixtures contained 5 μ l of extracted nucleic acids, 0.5 μ M of each primer, 250 μ M of each type of deoxyribonucleotide triphosphate, 10 μ l of 5 $\times$ GoTaq[®] Flexi Buffer, 1.25 U GoTaq[®] DNA polymerase, 1.5 mM MgCl₂ (Promega Corporation, Madison, WI, USA) and sterile MilliQ water for a final volume of 50 μ l. PCR amplification was performed using the following program: 95 °C for 2 min, 20 cycles of denaturation at 92 °C for 45 s, annealing at 50 °C for 2 min, and extension at 72 °C for 1 min and 45 s, and a single final extension at 72 °C for 5 min. For the secondary amplification, the primer pair 341F-GC (with 40 bp GC-clamp) and 907R (Chong *et al.*, 2009) was used. The reaction mixtures consisted of 2 μ l of template, 0.5 μ M of each primer, 400 μ M of each deoxyribonucleotide triphosphate, 10 μ l of 5 $\times$ GoTaq[®] Flexi Buffer, 1.25U GoTaq[®] DNA Polymerase, 1.5 mM MgCl₂ (Promega Corporation) and sterile MilliQ water for a final volume of 50 μ l. Touchdown PCR was performed with an initial denaturation step of 94 °C for 5 min, 10 touchdown cycles of 94 °C for 1 min, 65 °C (-1 °C per cycle) for 1 min and 72 °C for 3 min, 5 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final elongation step of 72 °C for 4 min. The presence of PCR products was confirmed by analysing 5 μ l of product on 1.5% agarose gels and staining with ethidium bromide.

Denaturing gradient gel electrophoresis (DGGE) was performed with the BIO-RAD DCode™ Universal Mutation

Detection System. PCR samples were loaded onto 7% (w/v) polyacrylamide gels in 1× TAE (40 mM Tris base, 20 mM acetic acid, glacial, 1 mM EDTA [pH 8.0]). The 7% (w/v) polyacrylamide gels (acrylamide/Bis solution, 37.5:1; BIO-RAD Laboratories, Inc., Hercules, CA, USA) were made with denaturing gradients ranging from 35% to 60% (where the 100% denaturant contains 7 M of urea and 40% deionised formamide). The electrophoresis was performed at 60 °C for 16 h at 70 V. After electrophoresis, the gels were soaked for 15 min in 250 ml of 1× TAE running buffer and 0.5 µg/ml ethidium bromide, rinsed for 15 min in 250 ml of 1× TAE running buffer and photographed.

DGGE patterns were analysed using the FPQuest Software Version 5.1 (BIO-RAD Laboratories, Inc.) to generate a band-matching table. All bands are divided into classes of common bands and for each pattern a particular band class can have two states: present or absent (binary matrix). The binary matrix obtained was exported to perform the Principal Component Analysis (PCA) with the PAST software (Hammer *et al.*, 2001).

Statistical analysis

Data are reported as means with their standard errors (SEM). Statistical analyses were performed by one way ANOVA followed by the Tukey post-test. The Mann-Whitney U-test was applied to the PCA scores in order to evaluate the effect of the oral administration of a daily dose of 1×10^{10} cells of *B. indicus* HU16 on gut microbiota of rats under a high fat diet. Probability values less than 0.05 were considered to indicate a significant difference. All analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

Materials

All chemicals used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3. Results

Cells but not spores of *Bacillus indicus* HU16 rescued early signs of diet-induced metabolic syndrome

After 4 weeks of dietary treatment, insulin tolerance tests were carried out to screen the efficacy of the treatment with spores or cells. The cumulative change in plasma glucose after insulin injection assessed through the measurement of area under the curve indicated that after 4 weeks of dietary treatment, high fat fed rats had already developed insulin resistance (Figure 1). The same effect was evident also in high fat fed rats treated with HU16 spores, while those that had received HU16 cells exhibited a glucose response to insulin very similar to that of low fat fed rats

and were, therefore, protected by the diet-induced insulin resistance (Figure 1).

Since only cell-treated rats showed an improvement in glucose homeostasis compared to high fat fed rats, the dietary treatment was continued up to 8 weeks only for low fat-fed (Group 1), high fat-fed (Group 2) and high fat-fed rats receiving *B. indicus* cells (Group 3).

Cells of *Bacillus indicus* HU16 did not affect obesity markers

High fat dietary consumption sustained the development of diet-induced obesity, as shown by: (1) the significant increase in body weight and body lipids, with a concomitant decrease in body water (Table 3); (2) the altered plasma lipid profile, with a significant increase in triglycerides, cholesterol and NEFA (Table 4); and (3) the increased deposition of triglycerides in the liver (Table 4). All these obesity markers were not affected by the daily treatment with 1×10^{10} cells of *B. indicus* HU16 (Table 3 and 4).

Figure 1. Plasma glucose levels (A) and area under the curve (AUC, B) during insulin tolerance test after 4 weeks of dietary treatment in rats fed low fat diet, high fat diet, high fat diet + spores and high fat diet + cells. Values are reported as mean values $\pm$ standard error of the mean; $n=6$ different rats. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ (one-way ANOVA followed by Tukey post-test).

Table 3. Body composition and energy balance.¹

	Low fat	High fat	High fat + cells
Initial body weight, g	475±19	473±15	478±15
Final body weight, g	561±16	599±12*	606±13*
Body weight gain, g	86±5	126±12*	128±14*
Body lipids, g/100 g bw	13.9±0.9	17.0±1.4*	18.6±1.3*
Body proteins, g/100 g bw	18.3±2.4	18.5±0.8	18.4±2.6
Body water, g/100 g bw	62.4±0.8	59.4±1.1*	57.2±1.8*
Epididymal adipose tissue, mg/g bw	16±1	22±2*	22±2*
Body energy, kJ/g	9.8±0.2	11.1±0.4*	11.6±0.3*
Metabolisable energy intake, kJ	11,931±189	12,278±355	12,099±287
Energy gain, kJ	1,473±89	2,615±367*	2,704±125*
Lipid gain, kJ	1,164±190	2,307±349*	2,310±215*
Protein gain, kJ	298±10	329±28	300±15
Energy expenditure, kJ	10,558±241	9,463±392	9,480±250

¹ Values are reported as mean values ± standard error of the mean; bw = body weight; n=6 different rats. * $P<0.05$ compared to low fat (one-way ANOVA followed by Tukey post-test).

Table 4. Plasma and liver parameters.^{1,2}

	Low fat	High fat	High fat + cells
Plasma triglycerides, mg/100 ml	97.6±5	204.2±10*	193.0±12*
Plasma cholesterol, mg/100 ml	57.1±2	105.9±10*	93.1±5*
Plasma non esterified fatty acids, mM	0.39±0.02	0.50±0.02*	0.46±0.02*
Plasma lipid peroxidation, nmol TBARS/ml	2.1±0.2	3.8±0.3*	2.8±0.2#
Plasma ALT, U/l	11.6±0.4	28.1±1.0*	17.8±1.0#
Plasma LPS, EU/ml	0.59±0.02	1.76±0.2*	1.0±0.1#
Plasma TNF-α, pg/ml	23.5±1.8	40.8±4.8*	25.5±1.7#
Hepatic triglycerides, mg/g tissue	10.2±2.3	24.5±2.7*	22.9±2.7*
Hepatic lipid peroxidation, nmol TBARS/g tissue	64.4±4.3	79.8±1.9*	68.6±2.6#
Hepatic mitochondrial lipid peroxidation, nmol TBARS/mg protein	0.52±0.01	0.60±0.02*	0.50±0.01#

¹ ALT = alanine aminotransferase; LPS = lipopolysaccharide; TBARS = thiobarbituric acid reactive substances; TNF = tumour necrosis factor.

² Values are reported as mean values ± standard error of the mean; n=6 different rats. * $P<0.05$ compared to low fat, # $P<0.05$ compared to high fat (one-way ANOVA followed by Tukey post-test).

Cells of *Bacillus indicus* HU16 rescued signs of diet-induced metabolic syndrome and oxidative damage

As previously reported (Crescenzo *et al.*, 2008), at the end of the 8-week period, high fat fed rats exhibited whole body insulin resistance (Figure 2). In fact, the decrease in plasma glucose concentration induced by a given dose of insulin was lower, and the increase in plasma insulin induced by a given dose of glucose was higher, in high fat fed rats compared to low fat fed rats (Figure 2). In both tests, the treatment with 1×10^{10} cells of *B. indicus* HU16 was protective against the development of insulin resistance (Figure 2).

Eight weeks of high fat diet also elicited a significant increase in systemic inflammation markers LPS and TNF-α, as well as an increase in oxidative damage of plasma lipids and hepatic necrosis (Table 4). These metabolic derangements were prevented by the administration of a daily dose of 1×10^{10} cells of *B. indicus* HU16 (Table 4). Oxidative stress was enhanced by eight weeks of high fat feeding in whole liver tissue and at the mitochondrial level (Table 4), but in this case the treatment of high fat fed rats with 1×10^{10} cells of *B. indicus* HU16 was protective (Table 4).

Eight weeks of treatment with HU16 cells did not affect the gut microbiota of high fat-fed rats

The majority of the samples from high fat fed animals treated (Group 3, circles) or not treated (Group 2, diamonds) with HU16 cells clustered together, while samples from two rats of each group were arranged separately (Figure 3). The Mann-Whitney U test performed on the PCA scores did not identify any statistically significant difference between the two groups. Therefore, the DGGE analysis suggests that the oral administration of a daily dose of 1×10^{10} cells of *B. indicus* HU16 did not affect the microbial composition of the animal gut.

4. Discussion

A high fat diet was used in the present study to mimic human diets that have been associated with the development of the metabolic syndrome. In fact, this diet induced changes in metabolic and hepatic functions, such as excessive abdominal fat deposition, impaired glucose tolerance and increased plasma lipid concentrations (Crescenzo *et al.*, 2008), together with increased plasma oxidative stress markers and liver transaminase compared with low-fat diet fed rats. On this animal model, we tested the hypothesis that the administration of the carotenoid-producing bacterial strain *B. indicus* HU16 could reverse obesity and/or associated metabolic syndrome induced by long-term dietary treatment with a high fat diet. At variance with results obtained with marine carotenoid fucoxanthin (Gammone and D'Orazio, 2015), simultaneous

Figure 2. Plasma glucose levels (A) and area under the curve (AUC, B) during insulin tolerance test, plasma insulin levels (C) and area under the curve (AUC, D) during glucose tolerance test, plasma glucose levels (E) and area under the curve (AUC, F) during glucose tolerance test, after 8 weeks of dietary treatment in rats fed low fat diet, high fat diet or high fat diet + cells. Values are reported as mean values $\pm$ standard error of the mean; $n=6$ different rats. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ (one-way ANOVA followed by Tukey post-test).

administration of carotenoid-producing bacterial cells did not affect the development of high fat diet-induced obesity nor abolished the modifications of plasma lipid profile. Interestingly, however, the most relevant high fat-associated metabolic impairment, i.e. insulin resistance, was completely reversed by bacterial administration. In addition, although plasma lipids were increased in high fat fed rats receiving bacterial cells, this treatment abolished the increase in peroxidised lipids, suggesting that the

condition of increased oxidative stress elicited by high fat diet is prevented by bacterial administration. This result is interesting, since oxidative stress is today considered a leading cause for the onset of obesity-associated insulin resistance (Bettaieb *et al.*, 2014; Rains and Jain, 2011).

Obesity is also associated with metabolic endotoxemia and a proinflammatory state, that contribute to the metabolic derangement observed both in human and animals (Cani

Figure 3. Principal component analysis score plot of PCR-DGGE patterns of gut microbiota of high fat-fed rats treated (black circles) and not treated (black triangles) with a daily dose of 1×10^{10} cells of *Bacillus indicus* HU16.

et al., 2007; Di Luccia *et al.*, 2015; Gomes *et al.*, 2017). We therefore looked at this aspect in our experiments to verify whether bacterial administration could exert an influence on the above parameters and we found a protective effect of these bacteria with regard to metabolic endotoxemia and inflammation. In addition, bacteria also exerted a protective effect on liver necrosis and therefore we looked at liver function more in depth. One important and deleterious consequence of high fat feeding and obesity is the development of hepatic triglyceride deposition, that represents the first step on the way to the development of non-alcoholic steatohepatitis, the second step being represented by oxidative stress (Asrih and Jornayvaz, 2015). High fat diet resulted in the increased triglyceride deposition, accompanied by increased lipid peroxidation in the liver and plasma of rats. The administration of *Bacillus* bacteria prevented these adverse effects of high fat diet and appears to be protective against oxidative stress. Mitochondria are primary cellular sites of production of reactive oxygen species and we looked at the oxidative damage in these organelles. Hepatic mitochondria isolated from rats fed a high fat diet showed a high degree of oxidative stress, while in rats fed a high fat diet and supplemented with bacteria, the degree of oxidative stress was found to be identical to that of rats fed low fat diet, thus suggesting that bacterial supplementation exerts a protective role also on mitochondria.

It is well established that the diet is a main factor involved in shaping the microbial composition of the gut (De Filippo *et al.*, 2010) and that a high fat diet alters the gut microbiota both in animal models (Di Luccia *et al.*, 2015; Rabot *et al.*, 2016) and in humans (Sonnenburg and Backed, 2016). Such alterations have been linked to the development of the metabolic syndrome and obesity (Murphy *et al.*, 2015). We observed by DGGE analysis that the oral administration of a daily dose of 1×10^{10} cells of *B. indicus* HU16 to high fat fed rats did not significantly alter the microbial composition of the gut. Although a metagenomic approach would be

needed to completely rule out the possibility that HU16 cells had an effect on the gut microbiota, our DGGE analysis suggests that major alterations in the gut composition had not occurred. Based on this, we hypothesise that HU16 cells produce metabolites able to directly reduce the oxidative damages caused by the high fat diet. Because of the well-known anti-oxidant activity of carotenoids, the carotenoids produced by HU16 cells are an obvious candidate. Verifying this hypothesis will require new *in vivo* experiments with the purified carotenoid and a HU16 mutant unable to produce the carotenoid and will be a challenging task for future work. An additional question raised by this work is why HU16 spores do not show any beneficial effect in our rat model of diet-induced metabolic syndrome. It has been elegantly demonstrated that *B. subtilis* spores once ingested can perform an entire life cycle in the mouse gastrointestinal tract, germinating in the upper part of the intestine, undergoing few rounds of growth and then sporulating the lower intestine (Casula and Cutting, 2002). A similar evidence is not currently available for either the rat gastrointestinal tract or the *B. indicus* spore. Therefore, we hypothesise that in the rat intestine HU16 spores either: (1) do not germinate; (2) germinate with a very low efficiency; or (3) that germinated spores are unable to grow. According to this hypothesis cells but not spores have a beneficial, probiotic role. Further work will be needed to address this hypothesis.

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