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Effects of chromium-enriched *Bacillus subtilis* KT260179 supplementation on growth performance, caecal microbiology, tissue chromium level, insulin receptor expression and plasma biochemical profile of mice under heat stress

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Abstract

The aim of this study was to investigate the effect of providing supplementary Cr-enriched *Bacillus subtilis* (CEBS) to mice with regard to their growth performance, caecal microbiology, tissue Cr concentration, insulin receptor (IR) expression and plasma biochemical profile. A total of ninety-six Kunming strain mice were allocated to four different groups: control, CEBS, inorganic Cr and *B. subtilis*. After 15 d of treatment, mice that received CEBS or normal *B. subtilis* had higher body weights than control mice, and after 30 d mice given either CEBS or *B. subtilis* had greater body weights than control mice or those given inorganic Cr. The concentration of Cr in tissues (heart, liver, spleen, kidney and skeletal muscle) increased after CEBS supplementation. *B. subtilis* and CEBS supplementation caused a significant increase in the numbers of *Lactobacillus* and *Bifidobacterium* in the caecum, whereas the numbers of *Escherichia coli* and *Staphylococcus* decreased significantly compared with the control. The levels of IR RNA and protein in skeletal muscles increased significantly. Plasma glucose, total cholesterol, TAG and LDL-cholesterol levels declined significantly in the CEBS group compared with the control group, whereas plasma insulin and HDL-cholesterol levels increased significantly. In conclusion, CEBS supplementation enhanced the regulation of body growth, increased tissue organic Cr concentrations, altered caecal microbiota and enhanced IR expression to produce significant changes in plasma biochemistry.

Key words: **Cr-enriched *Bacillus*: Mice: Metabolism: Gene expression: Heat stress**

Cr is an essential trace element and its beneficial effects on health are well documented in humans and other animals[1,2] – for example, the element is an integral component of the glucose tolerance factor[3] that influences the activity of insulin. Supplementation of the diet with trivalent Cr (Cr(III)) can be achieved using the salt $CrCl_3$. Other sources of Cr such as low molecular weight organic Cr complexes such as picolinic acid and nicotinate salt forms[4–6] provide a myriad of benefits with higher organic bioavailability than the inorganic forms[7] that are most often used as dietary supplements. Although Cr nanocomposites have even higher bioavailability than organic sources of Cr[8], their greater cost has inhibited widespread use. In humans and animal husbandry, there is a need to explore a cheap and convenient organic source of Cr for use in industrial applications.

In the south of China, summer temperatures can reach 35°C or more. Such high temperatures can cause heat stress to both humans and animals. It has been demonstrated that stress conditions increase the urinary excretion of Cr[9] and may exacerbate a marginal Cr deficiency[10]. It is also well known that stressful conditions may retard growth, depress food intake, change hormone release, increase disease susceptibility or lead to behavioural changes[11,12]. Some studies have reported that supplementation of the diet with Cr can alleviate the detrimental effects of heat stress[12,13]. Dietary Cr supplementation has also been shown to have a positive effect on growth performance and feed conversion ratio in poultry and dairy cows[14,15]. Whether dietary supplementation with Cr might play similar roles against heat stress in humans and other model animals needs further study.

Bacillus subtilis is a probiotic bacterium that is widely used in diets of both humans and animals[16,17]. Oral administration of *B. subtilis* can exert a range of beneficial effects including optimising the balance of intestinal microbiota, prevention and treatment of some diarrhoeal diseases, improvement of growth, enhancement of immune responses and reduction of serum cholesterol levels[18–20]. For these reasons, *B. subtilis* has attracted considerable attention as a potentially beneficial dietary supplement for human and animal health.

We thought that it might be worthwhile to explore whether the combined use of organic Cr and *B. subtilis* might have a greater effect on regulating body metabolism. To test this hypothesis, we grew Cr-enriched *B. subtilis* (CEBS), using certain concentrations of Cr, under the appropriate environmental

Abbreviations: ADG, average daily gain; CEBS, Cr-enriched *Bacillus subtilis*; F:G, ratio of feed:gain; GAPDH, glyceraldehyde phosphate dehydrogenase; IR, insulin receptor; TC, total cholesterol.

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conditions to enable the efficient conversion of inorganic Cr into organic forms. Thus, CEBS combines the virtues of *B. subtilis* and those of organic Cr and might induce an enhanced response to dietary supplementation. We tested CEBS using laboratory mice (Kunming strain), an experimental model in widespread use for such metabolic studies[21,22]. The ultimate aim of our study was to determine whether CEBS supplementation could play a role in modulating body growth, lipid metabolism in healthy mice in summer conditions and might be of use for preventing heat stress-related metabolic diseases in humans and animals.

Methods

Generation of chromium-enriched Bacillus subtilis

B. subtilis was provided by the Institute of Animal Husbandry and Veterinary Medicine, Anhui Academy of Agricultural Sciences in China. We grew the cells in medium containing $CrCl_3{\cdot}6H_2O$ (Cr concentration 30 μg/ml). After culture for 36 h, the cells were harvested when the live *B. subtilis* reached $1{\cdot}0 \times 10^9$ colony-forming unit (CFU)/ml. Subsequently, cell-free culture supernatant (CFCS) was prepared by centrifuging 50 ml of the culture medium for 15 min at 3000 ***g*** and sterilising by filtration through a 0·45-μm membrane filter (Millipore Corporation). The precipitate from the centrifugation step was also collected. The Cr concentrations of the medium, CFCS and precipitate were 30 μg/ml, 1·2 μg/ml and 1438·3 μg/g, respectively. The Cr in the CFCS was mainly in the inorganic form, whereas in the precipitate it was mainly in the organic form. The rate of conversion to organic Cr was 96 %. In order to determine the Cr form, we extracted proteins and nucleic acids from the CEBS and measured the concentration of Cr bound to these molecules. The analysis indicated that 90·86 % of total organic Cr was bound to proteins, whereas 6·37 % of Cr was bound to nucleic acids. We concluded that the Cr in CEBS was mainly in the organic form.

Animals and groups

A total of ninety-six male mice (Kunming strain) with an initial live average body mass of 11·5 g were obtained from Anhui Provincial Hospital, Anhui, China. Mice were quarantined for a minimum of 5 d before testing. All animals were asymptomatic and were released from quarantine before the start of the study. Mice were housed in clean cages. The feed duration was 30 d from 5 July to 4 August. The ninety-six mice were divided randomly into four groups, each with three replicates of eight animals: group I was fed common basal feedstuff and given clean water (the concentration of Cr was 0·06 μg/ml); group II received basal feedstuff and water supplemented with CEBS (0·30 μg Cr/ml, 10^7 CFU/ml live *B. subtilis*); group III received basal feedstuff and water supplemented with 1·537 μg/ml $CrCl_3{\cdot}6H_2O$ (0·30 μg Cr/ml); and group IV received basal feedstuff and water supplemented with 10^7 CFU/ml *B. subtilis*. Supplementation with CEBS and normal *B. subtilis* was through drinking water by adding cells suspended in the fermented medium. The use of animals for this research was approved by the Institution of Animal Science and Welfare of Anhui Province (number: IASWAP2014070518). The temperature of the mouse facility was 33 ± 3°C and the relative humidity was 55–60 %.

Growth performance and sample collection

Initial body weight and feed consumption were recorded. After 15 and 30 d, the body weights of the mice were obtained after an overnight fast. The change in body weight (average daily gain (ADG)) in relation to food intake (ratio of feed:gain (F:G)) was calculated as follows:

$$ADG = \text{body increase (g)/number of days},$$
$$F{:}G = \text{mass of food intake(g)/body increase (g)}.$$

After 30 d, ten mice from each treatment group were selected, fasted for 12 h and then tissues and blood samples were harvested under general halothane anaesthesia. All the blood samples were collected in 2·0-ml sterile heparinised tubes for plasma biochemical assays (described below). The heart, liver, kidney, spleen and skeletal muscles were collected with excess fat and veins carefully trimmed away; these tissues were frozen in liquid N_2 and stored at –80°C until analysis.

These mice were killed, and their caeca were removed under aseptic conditions. The tissues were stored in sterile plastic tubes in boxes packed with ice and were immediately sent to our laboratory for plate counting of micro-organisms[23] (eosin methylene blue agar for *Escherichia coli*, de Man, Rogosa and Sharpe agar for *Lactobacillus*, *Staphylococcus* plate-count agar for *Staphylococcus* and blood liver broth agar for *Bifidobacterium* using the pour plate method; the assays were repeated three times).

Chromium assay

A ZEEnit 700 P atomic absorption spectrometer (Analytik Jena) was used for assaying Cr levels in tissues. The analytical lines 357·869 nm for Cr were used; peak volume selected absorbance (PVSA, i.e., the integrated absorbance of the centre pixel (CP) only, or summated over three pixels around the line core (centre pixel plus the adjacent ones, CP71)) was used for signal evaluation, corresponding to a spectral interval of 2·3 pm (CP) for Cr at 428·972 nm. All the measurements were performed by the method described by Afridi *et al.*[24]. Samples (0·1–0·2 g) of the heart, liver, kidney, spleen and skeletal muscles and 0·2 g feedstuff from all four groups were placed in beakers and digested by adding 10 ml of a nitric acid–perchloric acid (HNO_3–$HClO_4$) mixture. The mixture was heated on a sand bath until fumes appeared (the temperature was controlled at 200°C by monitoring the sand) and the solution had mostly evaporated. After cooling, 5 ml HNO_3 was added, and the heating procedure was repeated at 180°C. The cooled remainder was made up to 10 ml with distilled water. Eight replicates were used for each group.

Determination of insulin receptor mRNA levels using quantitative real-time PCR

The following primers were used to amplify insulin receptor (IR): forward, 5′AACTCCCTGAAATGACAGTGAAGA3′, and

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reverse, 5′TGACTGAACACTAACCCGAACCT3′. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the internal control and was amplified using the primers: forward, 5′CCATCTTCCAGGAGCGAGAT3′, and reverse, 5′AAACATGGGGGCATCAGC3′.

Total RNA was isolated from skeletal muscle using TRIZOL (Invitrogen) according to the manufacturer's protocol. Dried RNA pellets were re-suspended in 40 μl diethylpyrocarbonate-treated water. The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm. The RNA was either used immediately or stored at −70°C before complementary DNA (cDNA) synthesis. First-strand cDNA was synthesised from 2 μg of total RNA using Oligo dT primers and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Synthesised cDNA was diluted to one-tenth concentration with sterile water and stored at −20°C before use.

Quantitative real-time PCR was performed on an ABI PRISM 7500 Detection System (Applied Biosystems). Amplification was performed using a 20-μl reaction mixture containing 10 μl 2 × SYBR Green I PCR Master Mix (Toyobo), 1 μl of diluted cDNA, 1 μl each primer (10 μmol/l) and 7 μl of PCR-grade water. The amplification procedure for IR and GAPDH consisted of a 95°C step for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 34 s and 72°C for 1 min and an extension step at 72°C for 7 min.

Western blotting

Total protein extracted from the muscles was harvested. The homogenate was centrifuged at 14 000 rpm for 20 min, and the supernatant containing the proteins was then collected. Total protein was estimated by the Bradford method. Using SDS-PAGE, proteins were separated after loading 120 μg/lane; the separated proteins were transferred onto a polyvinylidene fluoride membrane by overnight incubation with 5 % non-fat skimmed milk in Tris-buffered saline/0·1 % Tween 20 (TBST) buffer at 4°C. Non-specific binding sites were blocked. The membrane was washed 3 × 20 min times with TBST and then incubated with appropriate polyclonal primary antibodies: IRS-1 antibodies[25] (mouse monoclonal antibody, dilution 1:1000; Abcam Company) and GAPDH[26] (mouse monoclonal antibodies, dilution 1:1000; Abcam Company). The membrane was then washed 3 × 20 min with TBST and then incubated with an anti-rabbit secondary antibody for 1 h at room temperature, washed and then incubated with the substrate for 1 min. The membrane was exposed to HyBlot film (Denvill) for 30 s in a dark room. The densities of the bands were analysed by ImageAlpha software. The GAPDH signal in each sample was used to normalise the insulin signal.

Plasma hormone, glucose and lipid analyses

We centrifuged mouse blood at 3000 ***g*** for 10 min and collected the plasma. Mouse plasma insulin levels were determined using an ELISA kit (Nanjing Jiancheng Bioengineering Institute), and plasma glucose concentrations were measured by a colourimetric hexokinase glucose assay (Sigma Diagnostics). Plasma total cholesterol (TC), TAG, LDL-cholesterol and HDL-cholesterol concentrations were measured using the appropriate detection kits (Nanjing Jiancheng Bioengineering Institute).

Statistical analysis

Statistical analyses of the data were performed using SPSS 16.0 (SPSS Inc.). Data are presented as mean values with their standard errors. Differences between groups were compared using ANOVA. Differences between means were assessed by Tukey's honestly significant difference test of *post hoc* multiple comparisons. Data on body weight, ADG, average daily feed intake (ADFI) and F:G was statistically processed as repeated measurements. A P value <0·05 was considered statistically significant.

Results

Growth performance

The growth performance of mice in the different treatments is shown in Fig. 1–4. Mice that received CEBS or normal *B. subtilis* had higher body weights and ADG after 15 d than control mice or those that received inorganic Cr supplementation ($P < 0·05$). The index of F:G in CEBS- and *B. subtilis*-treated mice was significantly lower than those of the control ($P < 0·05$) at 15 d.

After 30 d, final body weights and ADG in the CEBS group were significantly higher compared with the control and the inorganic Cr-supplemented groups ($P < 0·01$). Mice in treatment group IV also had a higher final body weight and ADG than control mice ($P < 0·05$). Over the entire feeding duration, the F:G in the CEBS group was the lowest ($P < 0·01$). The F:G index of group IV was lower compared with the control and inorganic Cr groups ($P < 0·01$). There were no differences in the ADFI among the four groups either after 15 or after 30 d.

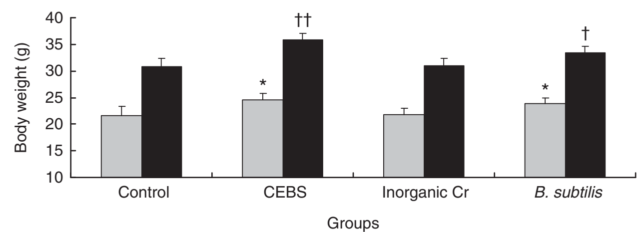

Fig. 1. Effects of different treatments on mice body weight. The mice were treated with control, Cr-enriched *Bacillus subtilis* (CEBS), inorganic Cr and *B. subtilis* after 15 and 30 d. Values are means, with standard errors represented by vertical bars. Data of body weight were statistically processed as repeated measurements. Mean value was significantly different from that of the control group: * $P < 0·05$, † $P < 0·05$, †† $P < 0·01$. ▒, 15 d; ■, 30 d.

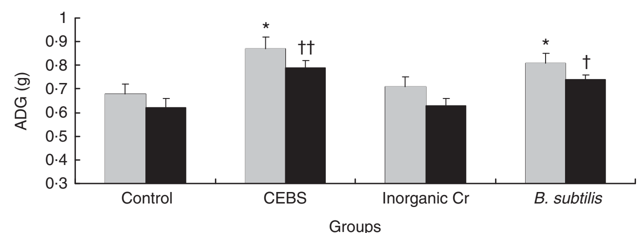

Fig. 2. Effects of different treatments on mice average daily gain (ADG). The mice were treated with control, Cr-enriched *Bacillus subtilis* (CEBS), inorganic Cr and *B. subtilis* after 15 and 30 d. Values are means, with standard errors represented by vertical bars. Data of ADG were statistically processed as repeated measurements. Mean value was significantly different from that of the control group: * $P < 0·05$, † $P < 0·05$, †† $P < 0·01$. ▒, 15 d; ■, 30 d.

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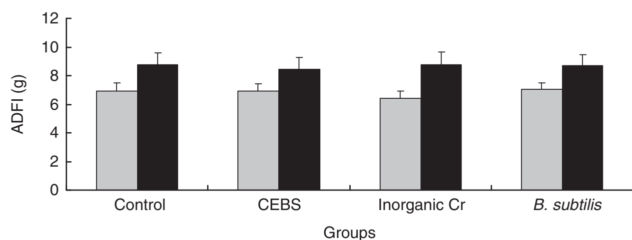

Fig. 3. Effects of different treatments on mice average daily feed intake (ADFI). The mice were treated with control, Cr-enriched *Bacillus subtilis* (CEBS), inorganic Cr and *B. subtilis* after 15 and 30 d. Values are means, with standard errors represented by vertical bars. Data of ADFI were statistically processed as repeated measurements. There were no differences among groups between 15 and 30 d ($P > 0{\cdot}05$). ▒, 15 d; ■, 30 d.

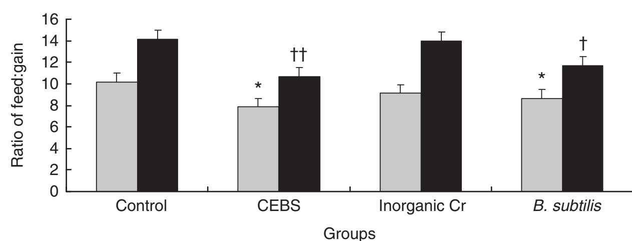

Fig. 4. Effects of different treatments on mice ratio of feed:gain (F:G). The mice were treated with control, Cr-enriched *Bacillus subtilis* (CEBS), inorganic Cr and *B. subtilis* after 15 and 30 d. Values are means, with standard errors represented by vertical bars. Data of ratio of F:G were statistically processed as repeated measurements. Mean value was significantly different from that of the control group: * $P < 0{\cdot}05$, † $P < 0{\cdot}05$, †† $P < 0{\cdot}01$. ▒, 15 d; ■, 30 d.

Caecal microflora

The caecal microflora in the different groups of mice was examined using the plate method (Table 1). Mice given CEBS or normal *B. subtilis* had lower numbers of *E. coli* and *Staphylococcus* compared with the control mice ($P < 0{\cdot}05$). There were no differences between the control and inorganic Cr-supplemented groups ($P > 0{\cdot}05$). The numbers of *Lactobacillus* and *Bifidobacterium* in the CEBS and normal *B. subtilis* groups increased significantly compared with the control and inorganic Cr-supplemented groups ($P < 0{\cdot}05$).

Tissue chromium concentrations

Cr concentrations in the heart, liver, spleen, kidney and skeletal muscles were measured (Table 2). The results indicated that mice fed inorganic Cr had significantly more Cr in these tissues compared with the control and *B. subtilis* supplementation groups ($P < 0{\cdot}01$). Mice in the CEBS group had the highest Cr concentrations in tissues, except for the spleen; for this organ, there was no significant difference with the inorganic Cr group.

Insulin receptor expression

The level of IR mRNA in skeletal muscles was measured (Fig. 5). Compared with control mice, there was a significant increase in IR mRNA in mice treated with inorganic Cr and CEBS. The difference between control and *B. subtilis* groups was not significant. Similar results were found for IR protein levels (Fig. 6) – that is, mice treated with inorganic Cr or CEBS had higher levels of IR protein.

Table 1. Effects of different treatments on caecal microflora ($\log_{10}$ colony-forming units/g)
(Mean values with their standard errors)

	Escherichia coli		*Staphylococcus*		*Lactobacillus*		*Bifidobacterium*	
Groups	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	8·48^{a}	0·13	8·39^{a}	0·12	9·01^{a}	0·15	8·04^{a}	0·07
CEBS	7·92^{b}	0·16	7·63^{b}	0·10	9·39^{b}	0·16	8·27^{b}	0·06
Inorganic Cr	8·46^{a}	0·15	8·29^{a}	0·13	9·03^{a}	0·16	8·06^{a}	0·08
Bacillus subtilis	7·88^{b}	0·13	7·88^{b}	0·11	9·37^{b}	0·13	8·21^{b}	0·07

CEBS, Cr-enriched *B. subtilis*.
a,b Mean values within a column with unlike superscript letters were significantly different ($P < 0{\cdot}05$).

Table 2. Effects of different treatments on tissue chromium content
(Mean values with their standard errors)

	Cr (ppb)									
	Heart		Liver		Spleen		Kidney		Skeletal muscle	
Groups	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	67·28^{Aa}	3·23	116·34^{A}	4·07	74·56^{Aa}	4·13	216·35^{Aa}	5·38	85·65^{A}	3·68
CEBS	82·37^{Cc}	3·26	146·38^{C}	4·33	94·33^{Bb}	4·79	273·85^{Cc}	5·27	123·64^{C}	3·17
Inorganic Cr	75·67^{Bb}	3·43	135·78^{B}	4·25	91·29^{Bb}	4·24	258·95^{Bb}	5·63	113·21^{B}	4·02
Bacillus. subtilis	66·83^{Aa}	3·38	114·96^{A}	4·02	73·28^{Aa}	4·71	217·41^{Aa}	5·49	86·31^{A}	3·27

ppb, Parts per billion; CEBS, Cr-enriched *B. subtilis*.
Aa,Bb,Cc Mean values within a column with unlike superscript letters were significantly different ($P < 0{\cdot}01$).

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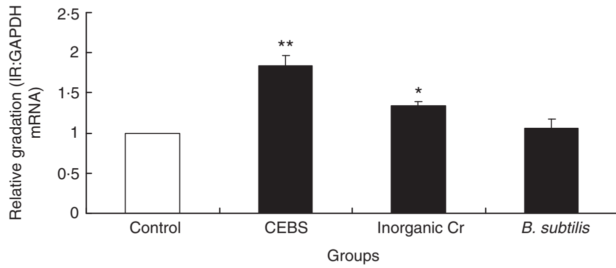

Fig. 5. Effects of different treatments on mRNA levels of insulin receptor (IR) in skeletal muscles. The mice were treated with control, Cr-enriched *Bacillus subtilis* (CEBS), inorganic Cr and *B. subtilis*. IR mRNA in skeletal muscles was measured by quantitative real-time PCR, and the ratio of the levels of IR mRNA:glyceraldehyde phosphate dehydrogenase (GAPDH) internal control was used for statistical comparison. Values are means, with standard errors are represented by vertical bars. Mean value was significantly different from that of the control group by one-way ANOVA followed by Tukey's multiple comparison tests: * $P<0{\cdot}05$, ** $P<0{\cdot}01$.

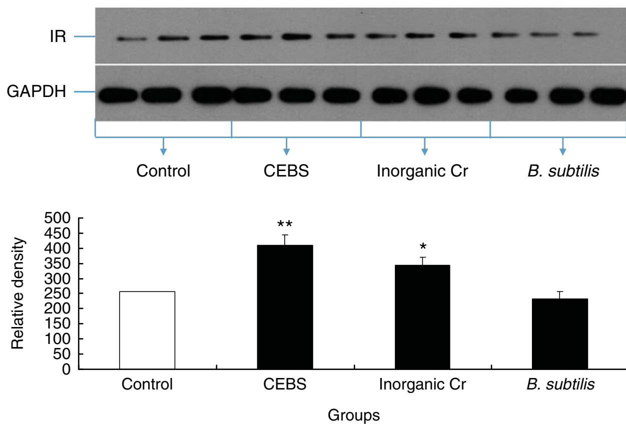

Fig. 6. Effects of different treatments on protein levels of insulin receptor (IR) in skeletal muscles. Mice were treated with control, Cr-enriched *Bacillus subtilis* (CEBS), inorganic Cr and *B. subtilis*. IR protein in skeletal muscles was measured by Western blotting, and the ratio of the levels of IR protein:glyceraldehyde phosphate dehydrogenase (GAPDH) internal control was used for statistical comparison. Values are means, with standard errors are represented by vertical bars. Mean value was significantly different from that of the control group by one-way ANOVA followed by Tukey's multiple comparison tests: * $P<0{\cdot}05$, ** $P<0{\cdot}01$.

Plasma insulin, glucose and lipids

Mice treated with inorganic Cr showed similar levels of plasma insulin as controls ($P>0{\cdot}05$; Table 3), whereas mice treated with CEBS showed enhanced insulin levels ($P<0{\cdot}01$) compared with controls. CEBS-treated mice also had the lowest plasma glucose levels ($P<0{\cdot}05$). Mice given inorganic Cr or *B. subtilis* had lower plasma glucose concentrations than the controls ($P<0{\cdot}05$). The levels of lipids (TC, TAG, LDL-cholesterol) in mice given inorganic Cr were significantly lower compared with the controls, but were still higher than those of the CEBS group ($P<0{\cdot}05$). The concentrations of HDL-cholesterol in the plasma of mice in the CEBS and inorganic Cr groups were higher compared with the control mice ($P<0{\cdot}05$), the former being higher in CEBS than inorganic Cr treatment ($P<0{\cdot}05$). The ratio of TC:LDL-cholesterol (TC:LDL-cholesterol) was calculated, which indicated that mice supplemented with CEBS had the highest ratio of all ($P<0{\cdot}01$). Mice supplied with inorganic Cr showed higher TC:LDL-cholesterol compared with the control and *B. subtilis* groups ($P<0{\cdot}01$). The results for the ratio of TC:HDL-cholesterol (TC:HDL-cholesterol) was opposite of that of TC:LDL-cholesterol. The ratio of the CEBS group was the lowest of all treatments ($P<0{\cdot}01$), and that for inorganic Cr was higher than that for CEBS but lower compared with the control and the *B. subtilis* treatments ($P<0{\cdot}01$).

Discussion

Although trivalent Cr (+3 oxidation state) is normally useable by organisms[27,28], under conditions of heat stress Cr is increasingly secreted from the body. Under stress conditions, supplementary Cr(III) may be necessary[29,30]. *B. subtilis* is often used as a probiotic and there are reports that it can be used to reduce heat stress in animals[31,32]. Our experiments indicated that rates of growth were improved in mice given CEBS or normal *B. subtilis* supplements, whereas there was no improvement after inorganic Cr supplementation. These results indicate that supplementary inorganic Cr cannot improve growth performance. Mice given CEBS had higher average body weights and greater feed utilisation efficiency than controls. The F:G index of the CEBS group was the lowest over the entire feeding period, suggesting that this treatment was more efficient than *B. subtilis* alone in regulating body growth performance.

Probiotic supplements have been reported to modify the composition of the caecal microbiota[33–35]. Similarly, our results indicated that *B. subtilis* supplements could alter the bacterial flora in the caeca of treated mice, whereas inorganic Cr did not have a significant effect compared with the control. In this study, 0·3 μg/ml $CrCl_3$ was added to the drinking water; this is an appropriate concentration for the treatment of animals[36,37]. The total Cr concentration in the CEBS treatment was 30 μg/ml; we therefore added a 1 % supplement to the water. The live *B. subtilis* reached 10^7 CFU/ml in the drinking water. Our results suggest that this supplementary dose of *B. subtilis* was suitable for the mice.

Previous studies have reported that Cr supplementation can increase the Cr content of tissues, although the results are varied[37–40]. In our study, the Cr contents of the heart, liver, spleen, kidney and skeletal muscles were significantly increased with the addition of Cr in the inorganic form and as CEBS. In rats, the increase in tissue Cr following Cr supplementation is the greatest in the kidney, followed by the liver, with considerably smaller changes in the heart and skeletal muscle tissues[38]. Our results showed that the supplemental Cr in CEBS had a significant influence on the Cr content of skeletal muscles in agreement with a previous report[37]. The effect of CEBS was greater than that for $CrCl_3$ in the present experiment, indicating that CEBS had greater bioavailability as an organic Cr resource.

Skeletal muscles account for 80 % of insulin-stimulated glucose disposal in the body[41,42]. Total RNA levels were increased in these studies by dietary supplementation with chromium picolinate. Therefore, we measured IR expression in skeletal muscles and found that total IR mRNA increased after 0·3 μg/ml inorganic Cr or after *B. subtilis* Cr supplementation. A previous study reported that 0·209 μg/ml Cr supplementation could increase insulin binding in cells and cause elevated IR

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Table 3. Effects of different treatments on plasma insulin, glucose and lipids
(Mean values with their standard errors)

	Insulin (mg/l)		Glucose (mmol/l)		TC (mmol/l)		TAG (mmol/l)		LDL-cholesterol (mmol/l)		TC:LDL-cholesterol		HDL-cholesterol (mmol/l)		TC:HDL-cholesterol	
Groups	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	2·25^{Aa}	0·23	6·87^{a}	0·23	2·71^{a}	0·13	1·19^{a}	0·05	1·58^{a}	0·06	1·72^{Aa}	0·06	0·63^{a}	0·03	4·30^{Aa}	0·13
CEBS	3·58^{Bb}	0·20	5·82^{c}	0·26	1·97^{c}	0·10	0·87^{c}	0·03	1·10^{c}	0·07	2·26^{Cc}	0·05	0·90^{c}	0·04	2·19^{Cc}	0·10
Inorganic Cr	2·45^{a}	0·25	6·25^{b}	0·23	2·35^{b}	0·12	0·93^{b}	0·02	1·26^{b}	0·04	1·86^{Bb}	0·04	0·71^{b}	0·02	3·31^{Bb}	0·14
Bacillus subtilis	2·37^{Aa}	0·21	6·32^{b}	0·21	2·69^{a}	0·11	1·23^{a}	0·04	1·61^{a}	0·05	1·67^{Aa}	0·05	0·61^{a}	0·03	4·41^{Aa}	0·12

TC, total cholesterol; TC:LDL-cholesterol, ratio of TC:LDL-cholesterol; TC:HDL-cholesterol, ratio of TC:HDL-cholesterol; CEBS, Cr-enriched *B. subtilis*.
^{a,b,c} Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).
^{Aa,Bb,Cc} Mean values within a column with unlike superscript letters were significantly different ($P<0·01$).

mRNA levels in muscle cells *in vivo*[43]. Our Western blotting analysis supported these results as IR protein levels increased after inorganic Cr or *B. subtilis* Cr supplementation. The amount of IR protein has been shown to increase after chromium picolinate supplementation in diabetic animal models and human patients[6,44–46]. Diabetic patients tend to lose the ability to convert Cr into a form that might potentiate insulin action. In our experimental conditions in which mice were maintained at 33°C, the loss of Cr led to a deficiency. Therefore, supplemental Cr could play a crucial role in regulating IR expression.

The level of serum glucose is regulated mainly by insulin. Under normal conditions, body glucose content returns to a normal level after dietary restriction or stress via the action of the glucose tolerance factor. Cr plays a part in the insulin signalling auto-amplification mechanism by stimulating IR kinase activity[47]. In the present study, we found that CEBS supplementation of drinking water increased the level of insulin and reduced plasma glucose levels. Likewise, supplementary inorganic Cr and *B. subtilis* reduced the plasma glucose levels and slightly increased insulin levels. Under heat stress conditions, supplemental Cr and *B. subtilis* might improve Cr metabolism and enhance the formation of glucose tolerance factor. TC in the body is regulated by the concentration of blood glucose and acetyl-CoA. An increase in fatty acids and a reduction in glucose mobilisation lead to a decrease in acetyl-CoA level, which regulates the synthesis of cholesterol. Our data suggested that the concentration of plasma TC and TAG were decreased in mice treated with inorganic Cr or *B. subtilis*. CEBS enhanced the metabolism of TC and TAG. HDL-cholesterol, synthesised mainly in the liver and the small intestine, plays an important part in eliminating serum cholesterol. The ratios of TC:HDL-cholesterol and TC:LDL-cholesterol are positively correlated with the incidence of CHD and atherosclerosis[48,49]. We found that supplemental inorganic and *B. subtilis* Cr could reduce plasma LDL-cholesterol levels and TC:HDL-cholesterol and increase HDL-cholesterol levels and TC:LDL-cholesterol. These results led us to conclude that supplementation with Cr may enhance the metabolism of cholesterol under heat stress conditions.

In conclusion, feeding supplementary CEBS combined the benefits of Cr and probiotics and altered body growth and caecal microbiota, tissue Cr concentrations, IR expression and plasma biochemical profile in mice maintained under heat stress.

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The contributions of the authors are as follows: J. Y. cultured and researched the CEBS, measured the mRNA and protein levels and wrote the paper. Y. X. fed the mice and recorded the growth data. K. Q. was involved in technical direction. W. Z. measured tissue Cr concentrations. D. W. measured the caecal microflora. C. W. analysed plasma hormones, glucose and lipids. The final manuscript was read and approved by all the authors.

The authors declare that they have no conflicts of interest.

References

1. McCarty MF (1982) Chromium and insulin. *Am J Clin Nutr* **36**, 384–385.
2. Mertz W (1993) Chromium in human nutrition: a review. *J Nutr* **123**, 626–633.
3. Lukaski HC (1999) Chromium as a supplement. *Annu Rev Nutr* **19**, 279–302.
4. Laschinsky N, Kottwitz K, Freund B, *et al.* (2012) Bioavailability of chromium(III)-supplements in rats and humans. *Biometals* **25**, 1051–1060.
5. Cefalu WT, Wang ZQ, Zhang XH, *et al.* (2002) Oral chromium picolinate improves carbohydrate and lipid metabolism and enhances skeletal muscle Glut-4 translocation in obese, hyperinsulinemic (JCR-LA corpulent) rats. *J Nutr* **132**, 1107–1114.
6. Wang ZQ, Zhang XH, Russell JC, *et al.* (2006) Chromium picolinate enhances skeletal muscle cellular insulin signaling *in vivo* in obese, insulin-resistant JCR:LA-cp rats. *J Nutr* **136**, 415–420.
7. Goodman S & Check E (2002) The great primate debate. *Nature* **417**, 684–687.
8. Eybe T, Audinot JN, Udelhoven T, *et al.* (2013) Determination of oral uptake and biodistribution of platinum and chromium by the garden snail (*Helix aspersa*) employing nano-secondary ion mass-spectrometry. *Chemosphere* **90**, 1829–1838.
9. Anderson RA, Bryden NA, Polansky MM, *et al.* (1988) Exercise effects on chromium excretion of trained and untrained men consuming a constant diet. *J Appl Physiol (1985)* **64**, 249–252.

https://doi.org/10.1017/S0007114515005127 Published online by Cambridge University Press

10. Rubin MA, Miller JP, Ryan AS, *et al.* (1998) Acute and chronic resistive exercise increase urinary chromium excretion in men as measured with an enriched chromium stable isotope. *J Nutr* **128**, 73–78.
11. Xie J, Tang L, Lu L, *et al.* (2015) Effects of acute and chronic heat stress on plasma metabolites, hormones and oxidant status in restrictedly fed broiler breeders. *Poult Sci* **94**, 1635–1644.
12. Toghyani M, Shivazad M, Gheisari A, *et al.* (2012) Chromium supplementation can alleviate the negative effects of heat stress on growth performance, carcass traits, and meat lipid oxidation of broiler chicks without any adverse impacts on blood constituents. *Biol Trace Elem Res* **146**, 171–180.
13. Sahin K, Ozbey O, Onderci M, *et al.* (2002) Chromium supplementation can alleviate negative effects of heat stress on egg production, egg quality and some serum metabolites of laying Japanese quail. *J Nutr* **132**, 1265–1268.
14. Leiva T, Cooke RF, Aboin AC, *et al.* (2014) Effects of excessive energy intake and supplementation with chromium propionate on insulin resistance parameters in nonlactating dairy cows. *J Anim Sci* **92**, 775–782.
15. Mirfendereski E & Jahanian R (2015) Effects of dietary organic chromium and vitamin C supplementation on performance, immune responses, blood metabolites, and stress status of laying hens subjected to high stocking density. *Poult Sci* **94**, 281–288.
16. Khochamit N, Siripornadulsil S, Sukon P, *et al.* (2015) Antibacterial activity and genotypic-phenotypic characteristics of bacteriocin-producing *Bacillus subtilis* KKU213: potential as a probiotic strain. *Microbiol Res* **170**, 36–50.
17. Park JH & Kim IH (2014) Supplemental effect of probiotic *Bacillus subtilis* B2A on productivity, organ weight, intestinal Salmonella microflora, and breast meat quality of growing broiler chicks. *Poult Sci* **93**, 2054–2059.
18. Jeong JS & Kim IH (2014) Effect of *Bacillus subtilis* C-3102 spores as a probiotic feed supplement on growth performance, noxious gas emission, and intestinal microflora in broilers. *Poult Sci* **93**, 3097–3103.
19. Kritas SK, Marubashi T, Filioussis G, *et al.* (2015) Reproductive performance of sows was improved by administration of a sporing bacillary probiotic (*Bacillus subtilis* C-3102). *J Anim Sci* **93**, 405–413.
20. Lei K, Li YL, Wang Y, *et al.* (2015) Effect of dietary supplementation of *Bacillus subtilis* B10 on biochemical and molecular parameters in the serum and liver of high-fat diet-induced obese mice. *J Zhejiang Univ Sci B* **16**, 487–495.
21. Li SG, Ding YS, Niu Q, *et al.* (2015) Grape seed proanthocyanidin extract alleviates arsenic-induced oxidative reproductive toxicity in male mice. *Biomed Environ Sci* **28**, 272–280.
22. Yang J, Huang K, Qin S, *et al.* (2009) Antibacterial action of selenium-enriched probiotics against pathogenic *Escherichia coli*. *Dig Dis Sci* **54**, 246–254.
23. Mountzouris KC, Tsirtsikos P, Kalamara E, *et al.* (2007) Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poult Sci* **86**, 309–317.
24. Afridi HI, Kazi TG, Talpur FN, *et al.* (2014) Evaluation of chromium and manganese in biological samples (scalp hair, blood and urine) of tuberculosis and diarrhea male human immunodeficiency virus patients. *Clin Lab* **60**, 1333–1341.
25. Liu HY, Han J, Cao SY, *et al.* (2009) Hepatic autophagy is suppressed in the presence of insulin resistance and hyperinsulinemia: inhibition of FoxO1-dependent expression of key autophagy genes by insulin. *J Biol Chem* **284**, 31484–31492.
26. Sakellariou GK, Davis CS, Shi Y, *et al.* (2014) Neuron-specific expression of CuZnSOD prevents the loss of muscle mass and function that occurs in homozygous CuZnSOD-knockout mice. *FASEB J* **28**, 1666–1681.
27. Schroeder HA (1968) The role of chromium in mammalian nutrition. *Am J Clin Nutr* **21**, 230–244.
28. Offenbacher EG & Pi-Sunyer FX (1988) Chromium in human nutrition. *Annu Rev Nutr* **8**, 543–563.
29. Moeini MM, Bahrami A, Ghazi S, *et al.* (2011) The effect of different levels of organic and inorganic chromium supplementation on production performance, carcass traits and some blood parameters of broiler chicken under heat stress condition. *Biol Trace Elem Res* **144**, 715–724.
30. Zhang FJ, Weng XG, Wang JF, *et al.* (2014) Effects of temperature-humidity index and chromium supplementation on antioxidant capacity, heat shock protein 72, and cytokine responses of lactating cows. *J Anim Sci* **92**, 3026–3034.
31. Deng W, Dong XF, Tong JM, *et al.* (2012) The probiotic *Bacillus licheniformis* ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. *Poult Sci* **91**, 575–582.
32. Moore T, Globa L, Pustovyy O, *et al.* (2014) Oral administration of *Bacillus subtilis* strain BSB3 can prevent heat stress-related adverse effects in rats. *J Appl Microbiol* **117**, 1463–1471.
33. Salazar N, Binetti A, Gueimonde M, *et al.* (2011) Safety and intestinal microbiota modulation by the exopolysaccharide-producing strains *Bifidobacterium animalis* IPLA R1 and *Bifidobacterium longum* IPLA E44 orally administered to Wistar rats. *Int J Food Microbiol* **144**, 342–351.
34. Sen S, Ingale SL, Kim YW, *et al.* (2012) Effect of supplementation of *Bacillus subtilis* LS 1-2 to broiler diets on growth performance, nutrient retention, caecal microbiology and small intestinal morphology. *Res Vet Sci* **93**, 264–268.
35. Shim YH, Ingale SL, Kim JS, *et al.* (2012) A multi-microbe probiotic formulation processed at low and high drying temperatures: effects on growth performance, nutrient retention and caecal microbiology of broilers. *Br Poult Sci* **53**, 482–490.
36. Jiajun Y, Aiyun H, Shanshan Z, *et al.* (2011) Regulation of organic nucleic acids and serum biochemistry parameters by dietary chromium picolinate supplementation in swine model. *J Trace Elem Med Biol* **25**, 91–96.
37. Zha LY, Xu ZR, Wang MQ, *et al.* (2007) Effects of chromium nanoparticle dosage on growth, body composition, serum hormones and tissue chromium in Sprague-Dawley rats. *J Zhejiang Univ Sci B* **8**, 323–330.
38. Prescha A, Krzysik M, Zablocka-Slowinska K, *et al.* (2014) Effects of exposure to dietary chromium on tissue mineral contents in rats fed diets with fiber. *Biol Trace Elem Res* **159**, 325–331.
39. Chang X & Mowat DN (1992) Supplemental chromium for stressed and growing feeder calves. *J Anim Sci* **70**, 559–565.
40. Uyanik F, Eren M, Guclu BK, *et al.* (2005) Effects of dietary chromium supplementation on performance, carcass traits, serum metabolites, and tissue chromium levels of Japanese quails. *Biol Trace Elem Res* **103**, 187–197.
41. Baron AD, Brechtel G, Wallace P, *et al.* (1988) Rates and tissue sites of non-insulin- and insulin-mediated glucose uptake in humans. *Am J Physiol* **255**, E769–E774.
42. Lang CH (1992) Rates and tissue sites of noninsulin- and insulin-mediated glucose uptake in diabetic rats. *Proc Soc Exp Biol Med* **199**, 81–87.
43. Qiao W, Peng Z, Wang Z, *et al.* (2009) Chromium improves glucose uptake and metabolism through upregulating the mRNA levels of IR, GLUT4, GS, and UCP3 in skeletal muscle cells. *Biol Trace Elem Res* **131**, 133–142.

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44. Jain SK, Kahlon G, Morehead L, *et al.* (2012) Effect of chromium dinicocysteinate supplementation on circulating levels of insulin, TNF-alpha, oxidative stress, and insulin resistance in type 2 diabetic subjects: randomized, double-blind, placebo-controlled study. *Mol Nutr Food Res* **56**, 1333–1341.
45. Sahin K, Tuzcu M, Orhan C, *et al.* (2013) Anti-diabetic activity of chromium picolinate and biotin in rats with type 2 diabetes induced by high-fat diet and streptozotocin. *Br J Nutr* **110**, 197–205.
46. Chen WY, Chen CJ, Liu CH, *et al.* (2009) Chromium supplementation enhances insulin signalling in skeletal muscle of obese KK/HlJ diabetic mice. *Diabetes Obes Metab* **11**, 293–303.
47. Davis CM & Vincent JB (1997) Chromium oligopeptide activates insulin receptor tyrosine kinase activity. *Biochemistry* **36**, 4382–4385.
48. Hofman A, Ott A, Breteler MM, *et al.* (1997) Atherosclerosis, apolipoprotein E, and prevalence of dementia and Alzheimer's disease in the Rotterdam Study. *Lancet* **349**, 151–154.
49. Walker SE, Register TC, Appt SE, *et al.* (2008) Plasma lipid-dependent and -independent effects of dietary soy protein and social status on atherogenesis in premenopausal monkeys: implications for postmenopausal atherosclerosis burden. *Menopause* **15**, 950–957.

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