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- (71) **Applicant (for all designated States except US):** GNOSIS SPA [IT/IT]; Piazza del Carmine, 4, I-20121 Milano (IT).
- (72) **Inventors; and**
- (75) **Inventors/Applicants (for US only):** CASTAGLIUOLO, Ignazio [IT/IT]; Gnosis SPA, Via Lavoratori Autobianchi, 1, I-20033 Desio (MI) (IT). BRUN, Paola [IT/IT]; Gnosis SPA, Via Lavoratori Autobianchi, 1, I-20033 Desio (MI) (IT). BUSIELLO, Immacolata [IT/IT]; Gnosis SPA, Via Lavoratori Autobianchi, 1, I-20033 Desio (MI) (IT). MIRAGLIA, Niccolò [IT/IT]; Gnosis SPA, Via Lavoratori Autobianchi, 1, I-20033 Desio (MI) (IT).
- (74) **Agent:** MINOJA, Fabrizio; Bianchetti Bracco Minoja S.r.l., Via Plinio, 63, I-20129 Milano (IT).

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(54) **Title:** FORMULATIONS CONTAINING *SACCHAROMYCES BOULARDII* AND SUPEROXIDE DISMUTASE (SOD) TO CONTROL OBESITY

(57) **Abstract:** Disclosed is a composition containing *Saccharomyces cerevisiae* var *boulardii* and the enzyme superoxide dismutase.



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**FORMULATIONS CONTAINING *SACCHAROMYCES BOULARDII* AND  
SUPEROXIDE DISMUTASE (SOD) TO CONTROL OBESITY**

The present invention relates to formulations useful as diet supplements containing the probiotic yeast *Saccharomyces boulardii* and the antioxidant enzyme superoxide dismutase. The formulations according to the invention are useful to reduce the risk of developing the metabolic syndrome correlated with obesity.

**State of the art**

Obesity is defined as a condition of excess body fat, and is associated with a large number of dysfunctions, including cardiovascular disease (CVD) and non-insulin-dependent diabetes mellitus (NIDDM).

The prevalence of obesity and excess weight in adults, children and adolescents has grown rapidly in the last 30 years and continues to increase, leading to high social costs.

Obesity is generally viewed as the result of a combination of an excessive energy intake and a sedentary lifestyle, but although these factors are naturally important, it has recently been suggested that sub-optimal intestinal flora also has an influence (Tennyson CA, Friedman G. Curr Opin Endocrinol Diabetes Obes. 2008 Oct;15(5):422-7), while systemic metabolic complications are associated with the low-grade systemic inflammation that arises in obese individuals (Fantuzzi G., J Allergy Clin. Immunol. 2005;115:911-919, Bäckhed F, Ding H, Wang T, PNAS 2004;101:15718-15723).

Moreover, it has been demonstrated that some species of bacteria, such as the lactic acid producing bacteria in the species *L. gasseri*, *L. casei* and *L. acidophilus*, are able to reduce the weight increase in rats fed on a carbohydrate-rich diet (Kang JH, Yun SI, Park HO, J Microbiol. 2010

Oct;48(5):712-4. Epub 2010 Nov 3).

In support of these studies, products containing probiotic bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* have been described which are able to prevent or cure metabolic dysfunctions deriving  
5 from obesity (WO 2010146568; WO 2010091992; US 2008267933).

To date, in addition to the well described properties of probiotic bacteria, many articles in the scientific literature have described some beneficial effects on the health of a probiotic yeast, such as the inactivation of *Clostridium difficile* toxin A by the yeast *Saccharomyces boulardii*  
10 (Castagliuolo I, Riegler MF, Valenick L, LaMont JT, Pothoulakis C.-Infect Immun. 1999 Jan;67(1):302-7) and the efficacy of *Saccharomyces boulardii* in preventing diarrhoea caused by antibiotic treatments or by infections due to intestinal pathogens (Surawicz CM, Elmer GW, Speelman P, McFarland LV, Chinn J, van Belle G.-Gastroenterology. 1989 Apr;96(4):981-8; McFarland  
15 LV- World J Gastroenterol. 2010 May 14;16(18):2202-22).

The enzyme superoxide dismutase (SOD), when administered parenterally, has proved effective in a number of inflammatory disorders (Carroll IM, et al. Am J Physiol Gastrointest Liver Physiol. 2007 Oct;293(4):G729-38; Oku T, et al. Inflamm Bowel Dis. 2006 Jul;12(7):630-  
20 40) reducing free radical formation and tissue damage.

Despite the number and detail of these studies, a composition based on probiotic yeasts which is able to prevent or counteract obesity and the correlated disorders has never been studied or proposed.

### **Description of the invention**

25 The present invention relates to a composition containing a probiotic yeast and an antioxidant, which is useful to reduce the risk of developing obesity and the metabolic syndrome correlated with it.

The probiotic yeast present in the composition, which is able to reduce

the risk of developing the metabolic syndrome correlated with obesity, belongs to the genus *Saccharomyces*, and more preferably to the species *Saccharomyces cerevisiae* var. *boulardii* (hereinafter called Sb).

The antioxidant substance used in combination with the probiotic yeast  
5 Sb belongs to the class of antioxidant enzymes, and is more preferably the enzyme superoxide dismutase (hereinafter called SOD).

SOD is preferably prepared in gastroprotected form by means of conventional techniques and excipients. The effective dose of probiotic yeast is between  $10^6$  and  $10^9$  colony-forming units (CFU) per daily administration,  
10 preferably approx.  $10^7$  CFU, while the effective dose of SOD is between 1 and 100 mg, preferably between 2 and 50 mg, and more preferably between 5 and 20 mg a day. A dose of approx. 10 mg a day is particularly preferred.

The yeast and the SOD could also be administered simultaneously, separately or sequentially: in such case, the two active ingredients need not be  
15 associated in a single dose unit, and could optionally be used in a product in kit form comprising separate dosage forms for the two active ingredients.

The efficacy of the diet supplement supplied by this invention has been evaluated by monitoring physical properties such as weight, abdominal fat accumulation and food intake, and measuring some biochemical parameters  
20 such as blood glucose and the pro-inflammatory cytokine level.

The efficacy of the composition according to the invention as a diet supplement was tested on a non-genetic murine obesity model obtained in male mice of the C57BL/6 line, which had received from the age of 4 weeks a normal or high-calorie diet supplemented daily with Sb and SOD or with the  
25 carrier alone.

During the 9-week administration of the diet supplement, the body weight and food intake were evaluated every week.

At the end of the administration period, the animals underwent the

glucose tolerance test after fasting overnight; other animals were sacrificed to collect the peripheral blood needed to quantify the circulating cytokines and measure the accumulated abdominal fat.

The invention is described in greater detail in the examples below.

5       **Example 1:** Body weight, food intake and regulation of body fat.

In order to evaluate the efficacy of the diet supplement consisting of the composition of Sb and SOD in regulating the physical properties influenced by obesity, male C57BL/6 mice ( $15.2 \pm 0.3$  g) aged 4 weeks were divided into randomised groups to which a normal calorie diet (77% carbohydrate, 19% protein and 4% fat) or a high-calorie diet (VHF, 21% carbohydrate, 19% protein and 60% fat) was administered in the presence or absence of a daily supplement consisting of  $10^7$  CFU of Sb (administered by gastric probe) and/or 10 mg of SOD extracted from yeast, purified and made gastro-resistant by microencapsulation up to a specific activity of 180000 U/g, and incorporated in the food. However, other methods of gastroprotection, such as the gliadin film medium or other equivalent techniques, would produce the same effect and can therefore be considered equivalent for the purposes of the present invention.

Diet and food supplements were administered continuously for a total of 9 weeks.

20       The food intake of the animals (Table) and their body weight were monitored weekly, while the accumulation of visceral fat was quantified after 9 weeks' diet supplementation.

At the end of the experimental period the animals were sacrificed and the abdominal fat was carefully collected, weighed and then fixed in formalin for the subsequent histological tests (Figure 2a).

Diet supplementation with Sb + SOD, but not with Sb alone, significantly reduces the weight increase associated with a high-calorie diet (Figure 1). Moreover, the accumulation of adipose tissue at abdominal level

was significantly reduced in the animals that received Sb alone and in those that received Sb in combination with SOD (Figures 2 and 2a).

**Table**

| <b>Diet</b>         | <b>Supplement</b> | <b>Food intake per mouse per day (g)</b> | <b>Calorie intake per mouse per day (kcal)</b> |
|---------------------|-------------------|------------------------------------------|------------------------------------------------|
| Normal calorie diet | no                | 3,20                                     | 10,89                                          |
| High-calorie diet   | no                | 4,76                                     | 28,71                                          |
| High-calorie diet   | <i>Sb</i>         | 2,00                                     | 12,08                                          |
| High-calorie diet   | <i>Sb</i> + SOD   | 2,00                                     | 12,05                                          |

**Example 2:** Regulation of biochemical parameters in obesity.

In order to evaluate the efficacy of the diet supplement consisting of the composition of Sb and SOD in the metabolic complications associated with obesity after 9 weeks of the experiment described in example 1, the mice were subjected to an oral glucose loading test and a study of the cascade of signals induced by insulin in a target organ (liver).

To perform the oral glucose tolerance test, the blood glucose was determined after 18 hours' fasting and before administration of an oral glucose load (2 mg/g of body weight).

The blood glucose was then measured every 30 minutes for 120 minutes, and glucose tolerance was calculated as the area under the curve (AUC) (Figure 3a and Figure 3b).

To evaluate the functional state of the cascade of signals induced by insulin, an insulin load was administered intraperitoneally to the mice (10 U/Kg of weight). In this case the animals were sacrificed 5 minutes later, and liver samples were collected and frozen.

The total proteins were extracted from the liver samples and subjected

to the immunoblot test to evaluate the phosphorylation level of the IRS1 (Insulin Receptor Substrate 1) protein (Figure 4).

Under normal conditions, the bond between insulin and its receptor causes phosphorylation of the IRS1 protein on the Tyr941 residue, whereas in  
5 the case of the metabolic syndrome associated with obesity, this signal is reduced.

Supplementation with Sb or Sb + SOD using the same preparations and the same doses as in Example 1 reduced the extent of the post-glucose-load hyperglycaemia associated with obesity (Figures 3a and 3b).  
10 Co-administration of Sb and SOD caused a more marked reduction in post-load hyperglycaemia. Diet supplementation in obese mice with Sb and SOD restores the phosphorylation of IRS1 on the Tyr941 residue in response to a blood glucose load, therefore correcting the insensitivity of the target tissues to insulin (Figure 4).

15 **Example 3: Systemic inflammation caused by obesity.**

To evaluate the efficacy of the diet supplement consisting of the composition of Sb and SOD on systemic inflammation, a common condition in obese individuals, after 9 weeks' diet supplementation a sample of circulating blood was taken to measure the circulating levels of pro-inflammatory  
20 cytokine (TNF $\alpha$ ) and an adipokine (leptin). The assay was conducted on serum using the ELISA assay (Figure 5 and Figure 6).

In the case of obese animals an increase in the circulating levels of TNF $\alpha$  and leptin was observed, which contribute to the development of systemic complications. Diet supplementation with Sb or Sb + SOD using the  
25 same preparations and the same doses as in Example 1 significantly reduces the circulating levels of TNF $\alpha$  and leptin (Figure 5 and Figure 6). In particular, serum level of leptin, a pro-inflammatory adipokine derived from adipose tissue with a role in food taking regulation, is reduced by the

supplementation with Sb + SOD at a significantly higher rate even if compared to Sb and SOD single supplementations (Figure 6).

**Example 4:** Inflammatory state of the intestinal mucosa in obesity.

In order to evaluate the efficacy of the diet supplement consisting of the composition of Sb and SOD on the inflammatory condition of the mucosa associated with obesity after 9 weeks' diet supplementation, the animals were sacrificed so that a segment of ileum could be collected. The intestinal segments were homogenised in phosphate buffer to which a cocktail of protease inhibitors was added for the extraction of the total proteins. The homogenate was centrifuged at 13000x rpm for 10 min at 4°C, and the clarified supernatant was used to quantify the pro-inflammatory cytokines IL1- $\beta$  and TNF- $\alpha$  by ELISA assay and to quantify glutathione (GSH), the most effective anti-oxidant natural compound. The cytokine levels were normalised to the total protein levels determined by the Bradford assay.

In the case of obese individuals, an increase in the levels of IL1- $\beta$  and TNF $\alpha$ , which contribute to the development of systemic complications, is observed in the intestinal mucosa. Diet supplementation with Sb or co-administration of Sb and SOD using the same preparations and the same doses as in Example 1 induces a significant reduction in both cytokines (Figs. 7 and 8). Figures 7 and 8 clearly show that only co-administration of Sb and SOD restores IL1- $\beta$  and TNF $\alpha$  in the intestinal mucosa to comparable values with the control mice.

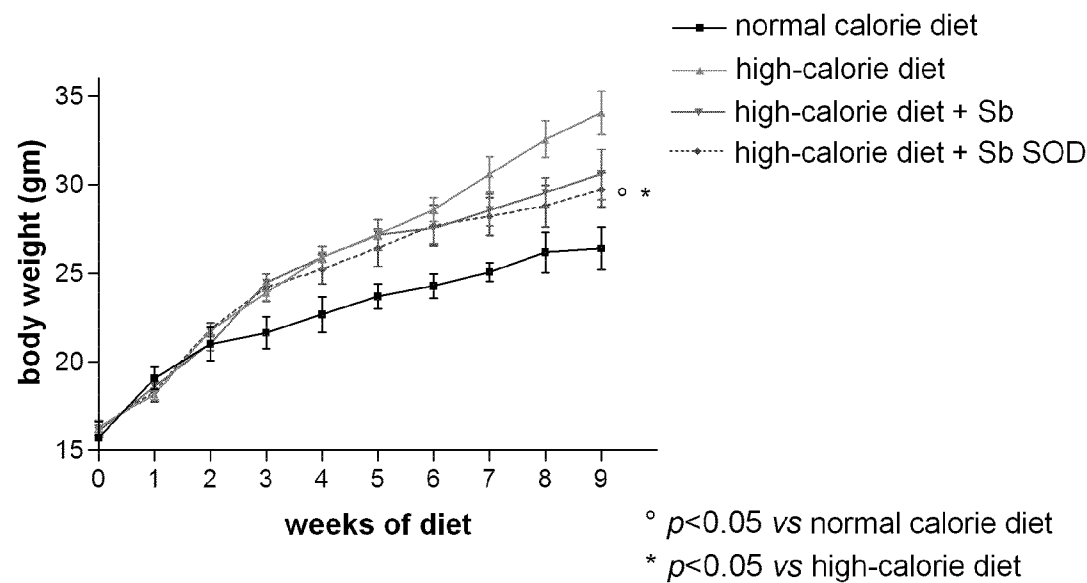
Glutathione (GSH), which has a powerful anti-oxidant activity and that can be related to an anti-inflammatory role at the mucosal level, shows a significant decrease in the ileum of the obese animals. Its level is surprisingly increased in case of a supplementation with Sb + SOD while single supplementations with either Sb or SOD alone are not effective (Figure 9).

**CLAIMS**

1. A composition containing *Saccharomyces cerevisiae* var *boulardii* and the superoxide dismutase enzyme.
- 5 2. A composition as claimed in claim 1 wherein the superoxide dismutase is in gastroprotected form.
3. A composition as claimed in claim 1 or 2 in kit form, suitable for the simultaneous, separate or sequential administration.
4. The composition of claim 1, 2 or 3 for use as a diet supplement.
- 10 5. The composition of claims 1 or 2 for use in the prevention or reduction of the risk of developing metabolic dysfunctions related to obesity.
6. The composition of claims 1 or 2 for use in the reduction of weight increase, for the reduction of food intake, for the reduction of body fat accumulation, and for the improvement of the systemic inflammatory
- 15 condition connected with obesity.

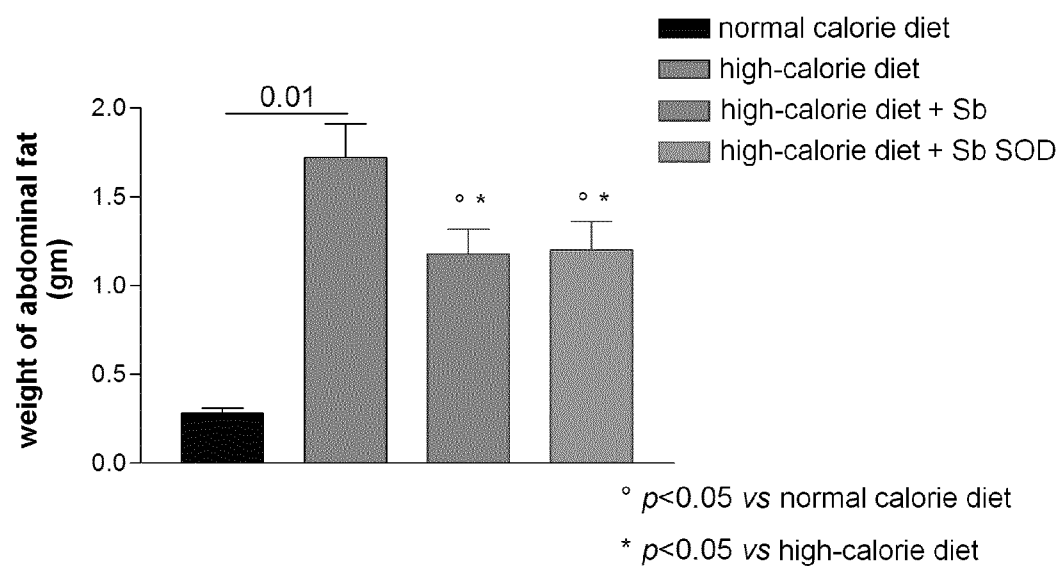
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Figure 1



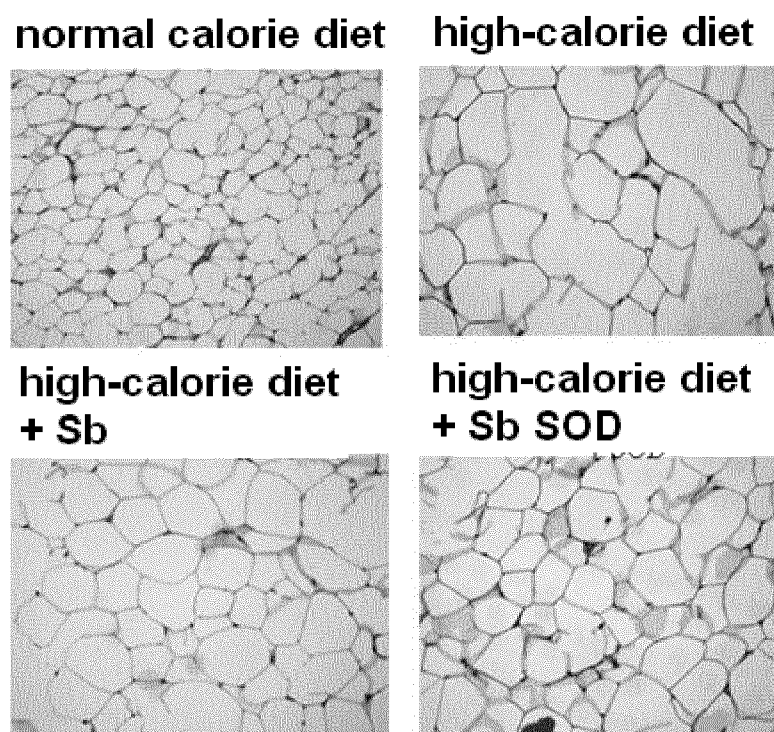
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Figure 2



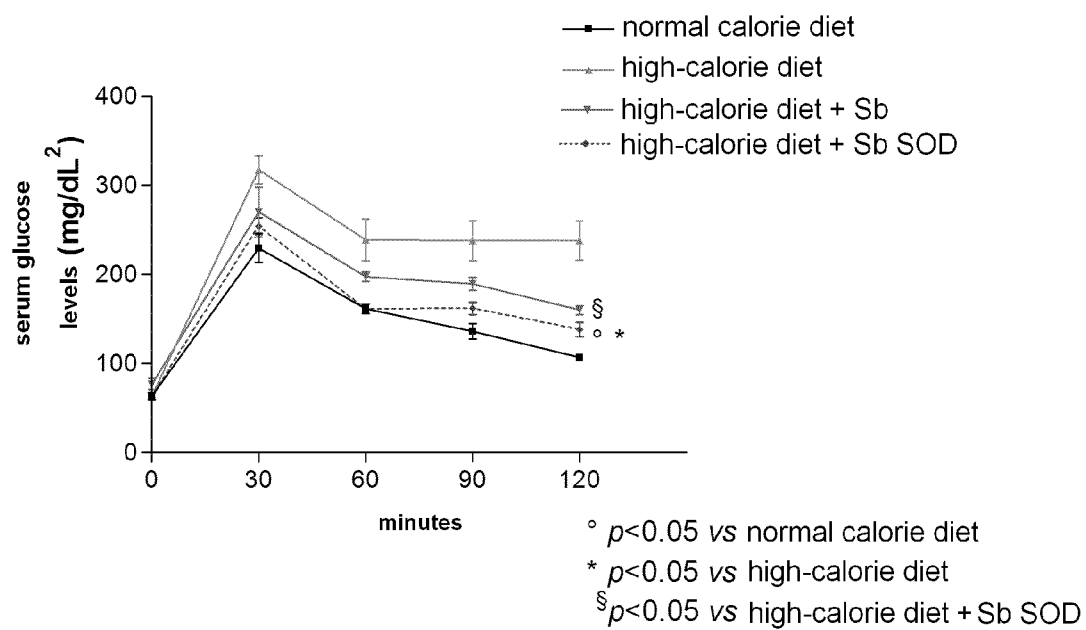
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Figure 2a



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Figure 3a



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Figure 3b

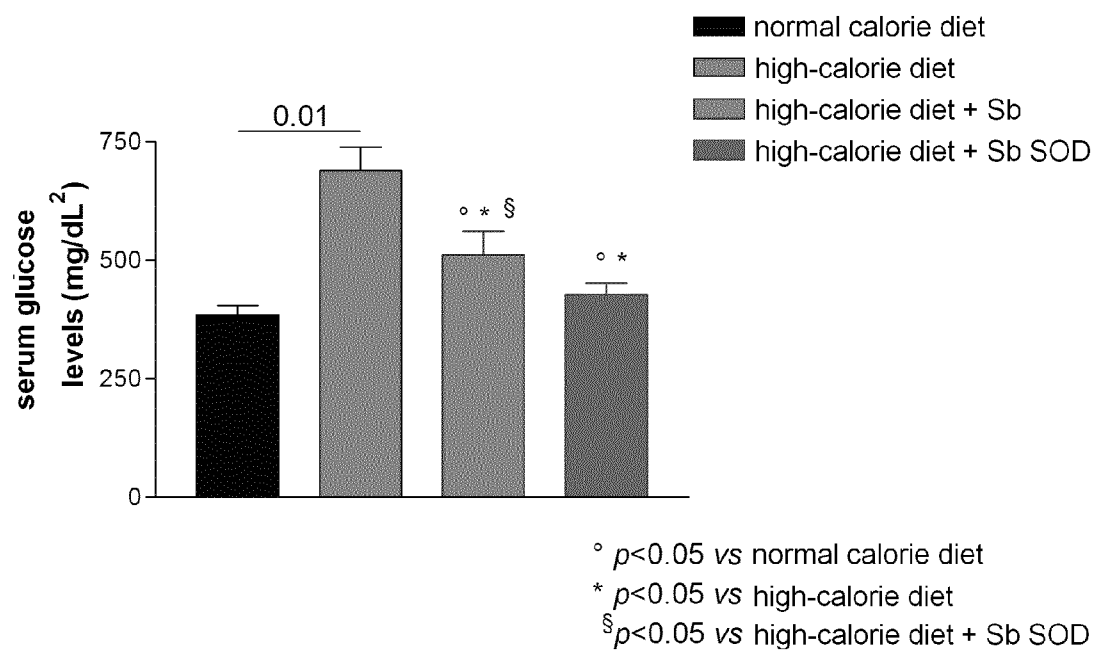
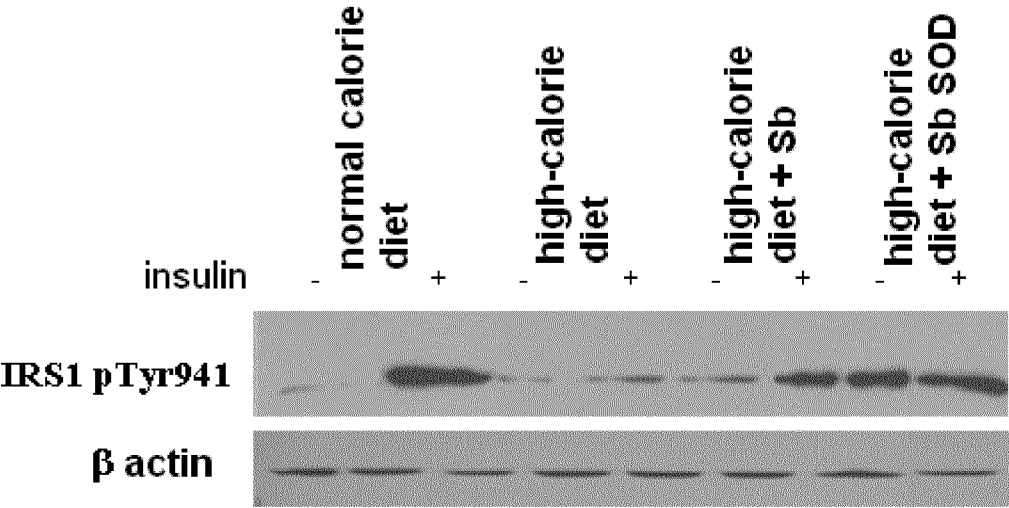
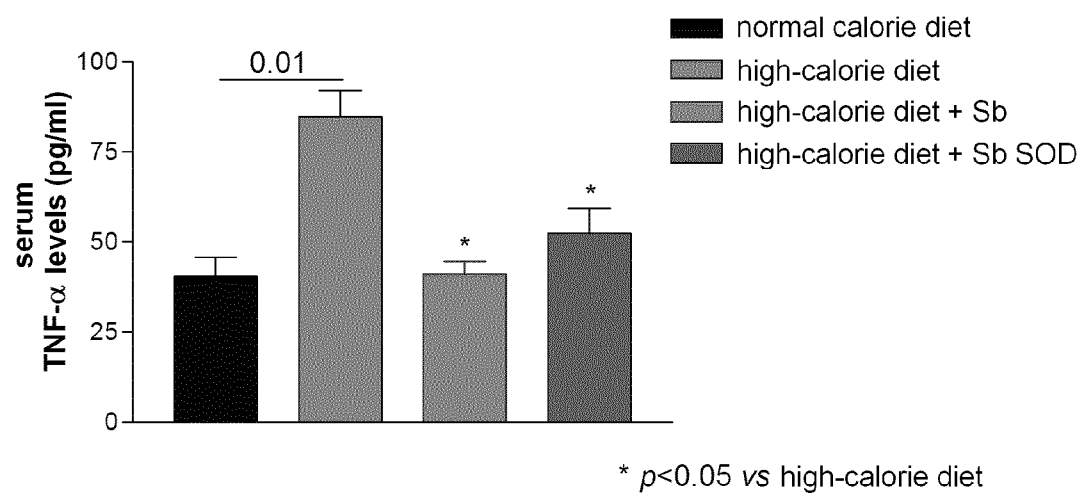


Figure 4

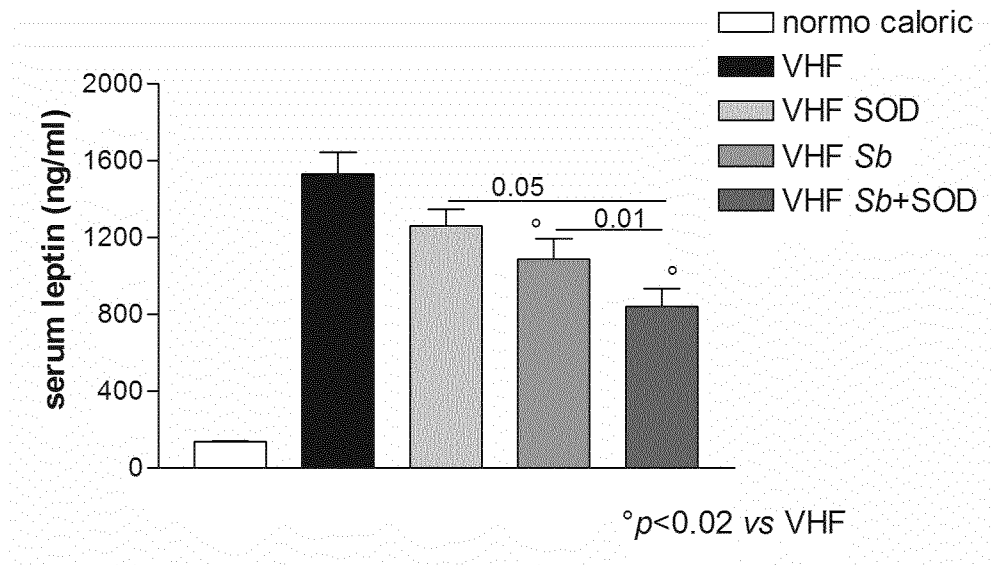


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Figure 5



### Figure 6

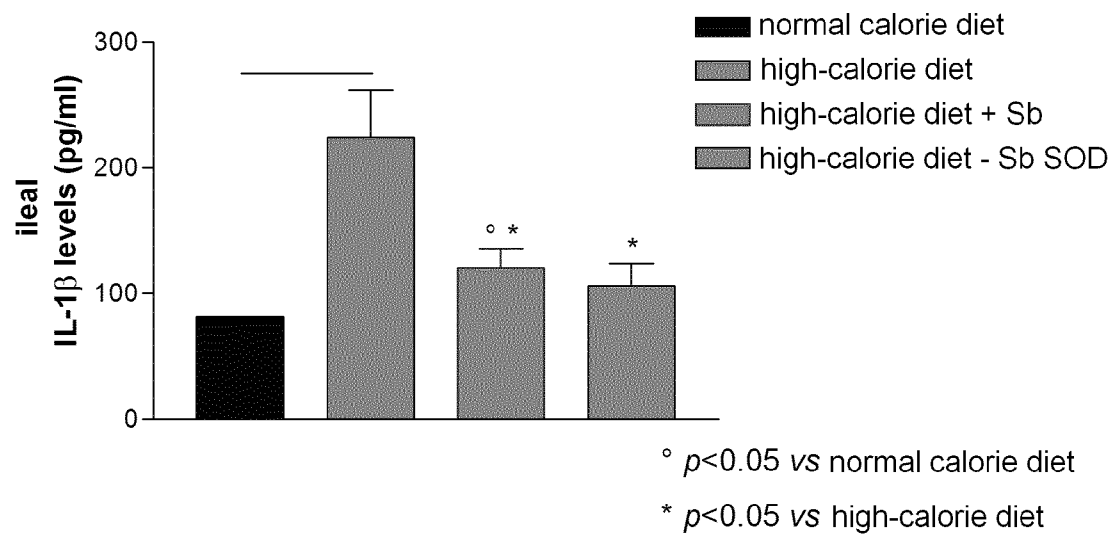


VHF Sb and VHF Sb+SOD  $p < 0.02$  vs VHF: ONE-way ANOVA with Bonferroni's post test

VHF SOD and VHF Sb vs VHF Sb+SOD: ONE-way ANOVA with Newman-Keuls' post test

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Figure 7



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Figure 8

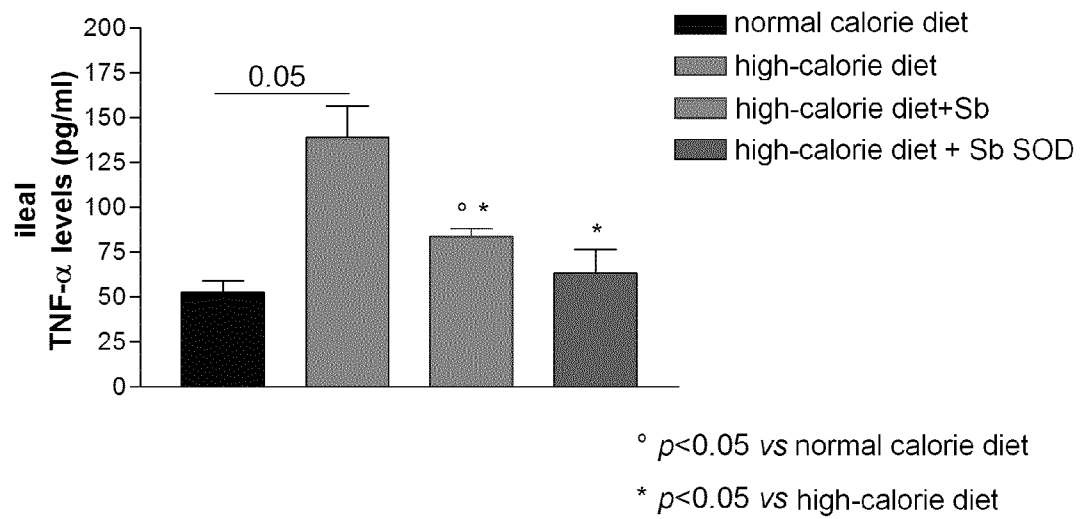


Figure 9

