Beneficial effects of fermented sardinelle protein hydrolysates on hypercaloric diet induced hyperglycemia, oxidative stress and deterioration of kidney function in wistar rats

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Abstract

This study investigated the potential effects of fermented sardinelle protein hydrolysates (FSPHs) obtained by two proteolytic bacteria, *Bacillus subtilis* A26 (FSPH-A26) and *Bacillus amyloliquefaciens* An6 (FSPH-An6), on hypercaloric diet (HCD) induced hyperglycemia and oxidative stress in rats. Effects of FSPHs on blood glucose levels, glucose tolerance, α-amylase activity and hepatic glycogen content were investigated, as well as their effect on the oxidative stress state. Biochemical findings revealed that, while undigested sardinelle proteins (USP) did not exhibit hypoglycemic activity, oral administration of FSPHs to HCD-fed rats reduced significantly α-amylase activity as well as glycemia and hepatic glycogen levels. Further, the treatment with FSPHs improved the redox status by decreasing the levels of lipid peroxidation products and increasing the activities of the antioxidant enzymes (superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT)) and the level of glutathione (GSH) in the liver and kidneys, as compared to those of HCD-fed rats. FSPHs were also found to exert significant protective effects on liver and kidney functions, evidenced by a marked decrease in alkaline phosphatase (ALP) level and a modulation of creatinine and uric acid contents. These results indicated the beneficial effect of FSPHs on the prevention from hyperglycemia and oxidative stress.

Keywords: Fermented sardinelle protein hydrolysates; Hypoglycemic; Antioxidant; Hepato-protective; Reno-protective.

Introduction

Diet is a key factor in the control of human health. In fact, HCD is the principal factor which causes the development of metabolic syndrome risks, defined as a cluster of the most dangerous heart attack risk factors including abdominal obesity, high cholesterol, hypertension, insulin resistance, type 2 diabetes mellitus T2DM (non-insulin-dependent diabetes mellitus NIDDM), etc (Grundy et al. 2004).

T2DM, known as the most common form of diabetes, is characterized by a persistent hyperglycemia. This hyperglycemic state represents one of the crucial factors for the development of oxidative stress and reactive oxygen species (ROS) (Yadav et al. 2000). It also results in severe micro and macro-vascular problems, such as the deficiency of many vital organs, especially eyes, kidneys, nerves, heart and blood vessels (Mahendran et al. 2014).

In addition, recent studies have demonstrated that detoxification organs, especially kidneys, represent a principal target to the action of oxidative stress development because of their high content of long-chain polyunsaturated fatty acids (Rodrigo and Rivera 2002). Free radical generation is also expected to induce liver damage, which is characterized by a progression from steatosis to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Srivastava and Shivanandappa 2010). Therefore, the use of antioxidants can be considred as the alternative method for chelation therapy. Accordingly, interest has recently grown in the role of natural antioxidants used as strategy to prevent oxidative damage as a factor in the pathophysiology of various health disorders (Shireen et al. 2008).

One of the therapeutic approaches for decreasing postprandial hyperglycemia is to retard absorption of glucose by inhibiting carbohydrate hydrolyzing enzymes such as α-amylase in the digestive organs (Heo et al. 2009). It was reported that many synthetic saccharides may reduce plasma glucose via retarding the liberation of glucose from dietary comlex

carbohydrates in the small intestine. However, the application of these inhibitors has often been reported to induce disturbances in the gastrointestinal tract, including flatulence, diarrhea and abdominal pain (Heo et al. 2009).

Interstingly, protein hydrolysates have been reported to present rich groups of peptides that exert interesting regulatory functions in humans, including hypolipidemic and antioxidative (Ben Khaled et al. 2012), ACE-inhibitory activities (Ktari et al. 2014), antidiabetic (Li-Chan et al. 2012), etc.

Fish proteins are of great interest for health human. In fact, fish meat is known as a source of high-quality proteins due to their high content in essential amino acids and nutritional value. In addition, several studies have used fish proteins as a matrix for the production of bioactive peptides and hydrolysates, able to prevent from various chronic diseases (Li-Chan et al. 2012; Ktari et al. 2014; Jemil et al. 2016a).

Although several protein hydrolysates have been produced by enzymatic hydrolysis (Ktari et al. 2014, Nasri et al. 2015), few studies have been conducted on the generation of biologically active peptides using microbial fermentation. In our previous study, we reported the production of protein hydrolysates from different fish species prepared by fermentation with *Bacillus subtilis* A26, exhibiting antioxidant activities (Jemil et al. 2014).

The present study investigated the potential effects of sardinelle protein hydrolysates obtained by fermentation treatment with *Bacillus subtilis* A26 and *Bacillus amyloliquefaciens* An6 with regards the modulation of hyperglycemia and the attenuation of oxidative stress, hepatotoxicity and renal damage in HCD-fed rats.

Materials and methods

Materials

Sardinelle (*Sardinella aurita*) was freshly purchased from the fish market of Sfax City, Tunisia. The sample was packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the laboratory within 30 min. Muscles were separated and then rinsed with cold distilled water to remove salts and other contaminants.

Sardinelle meat flour preparation

Sardinelle muscle (500 g) was homogenized in 1000 ml of distilled water and then cooked for 20 min at 100 °C. After removing bones from cooked fish, fillets were collected and dried in an oven at 80 °C for 18 h. The dried fish preparation was minced and the obtained powder was referred to the undigested sardinelle proteins (USP).

Production of fermented sardinelle protein hydrolysates

Sardinelle protein hydrolysates were prepared using *B. subtilis* A26 (CTM 50700) and *B. amyloliquefaciens* An6 as described in our previous work (Jemil et al. 2014). After 24 h of fermentation the degree of hydrolysis (DH) was measured using o-phthaldialdehyde (OPA) following the method referred by Nielsen et al. (2001). Each experiment was made in triplicate.

Chemical analysis

The proximate composition of USP and FSPHs was determined by evaluating the content of moisture and ash, according to the AOAC standard methods 930.15 and 942.05, respectively (AOAC 2000). The protein content was determined using the Kjeldahl method according to the AOAC method number 984.13 (AOAC 2000). A factor of 6.25 was used to convert the total nitrogen content into protein. Crude lipid content was determined after Soxhlet extraction, using hexane. All measurements were performed in triplicate.

Amino acid compositions of FSPHs

Amino acid compositions were determined after hydrolysis with 6M HCl at 110 °C for 24 h under N_2 atmosphere. Thereafter, the derivatization of hydrolysed amino acids was carried out using phenylisothiocyanate (Bidlingmeyer et al. 1984). Amino acids were separated using a reversed-phase high performance liquid chromatography (1200 Agilent Tech., CA, USA) equipped with a Pico Tag column (3.9 x 300 mm, 5 µm, Waters). Detection was performed at 254 nm, and amino acids were identified by their retention times compared to standards. The amino acid content was expressed as residue per 100 amino acid residues. All analyses were performed in duplicate.

Animals' treatment

A total of 30 male Wistar rats weighing about 150 g were purchased from the Central Pharmacy of Tunisia (SIPHAT, Tunisia). Rats were kept under controlled conditions of temperature (25±1 °C), relative humidity (60±5%), and light/dark cycle (12/12 h) in the laboratory of the Faculty of Sciences of Sfax city, Tunisia. Laboratory animal handling and experimental procedures were performed according to the guidelines of the Tunisian Ethical Committee for the care and use of laboratory animals. Before treatments, rats were fed a standard diet for a 1-week of acclimatization period.

The rats were divided into five groups of six animals each. Group 1, which served as control group, was rats fed a standard diet (Society of Animals Nutrition, Sfax, Tunisia). Detailed composition of the standard diet is illustrated in Jemil et al. (2016a). Group 2 contains rats fed a hypercaloric diet (HCD) during ten weeks. HCD was prepared by adding 10% sheep fat, 5% fructose and 0.1% cholic acid to standard diet, to induce hyperglycemia. Groups 3, 4 and 5 were rats fed HCD and received 400 mg/kg of body weight/day the USP,

FSPH-A26 and FSPH-An6, respectively. Treatments were administrated by oral gastric gavage.

Blood and tissues collection

After 10 weeks, wistar rats were sacrificed by decapitation. Blood samples were collected for further analyses. Detoxification organs (liver and kidneys) of each rat were carefully removed, weighed and then stored at −80 °C until use.

Samples from liver or kidney tissues were homogenized for 5 min in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), with a ratio of (1:2) (w/v) and then centrifuged at 9000 rpm for 15 min at 4 °C. The supernatants were collected and used for the various biochemical estimations.

Determination of hematological parameters

The Horiba ABX 80 Diagnostics (ABX pentra Montpellier, France) was used for the determination of hematological parameters including red blood cells (RBC) and its related indices following manufacturer's instruction. The determination includes hemoglobin (Hgb), hematocrit (Htc), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). White blood cells (WBC), platelets and lymphocytes were also analyzed.

Determination of serum glucose levels

The serum glucose levels in the different rat groups were determined using a commercial available kit (Glucose Oxidase-PAP, Biomaghreb, Tunisia).

Oral glucose tolerance test

In order to evaluate glucose tolerance of the different groups, animals received orally 2 g/kg glucose body weight, the day before sacrifice. Blood samples were collected from the tip of the tail of each animal before load (t=0) and 30, 60, 90 and 120 min after the glucose administration. Glycemia was measured using a glucometer (Bionime, GM550, Taichung city, Taiwan). Results were expressed as the integrated area under the curve of the blood glucose level (AUC glucose) which was calculated by the following trapezoid rule:

$$AUC\ glucose = \frac{C_1 + C_2}{2} \times (t_2 - t_1) \times 100$$

where, C_1 and C_2 are levels of plasma glucose at time t_1 and t_2, respectively; $t_2 - t_1 = 30$ min.

Determination of α-amylase activity in plasma

The α-amylase activity was evaluated using a commercial kit (Biolabo ref. 80023, Maizy city, France). The coloration intensity related to the α-amylase activity was measured at 405 nm and the enzymatic activity (U/l) in each serum was calculated using the following equation:

$$\text{α-amylase activity} = \frac{(\Delta \text{abs/min})_{\text{assay}}}{(\Delta \text{abs/min})_{\text{calibrator}}} \times \text{Calibrator concentration}$$

Where, $(\Delta \text{ abs/min})_{\text{Assay}}$: Rate of change per minute for the sample; $(\Delta \text{ abs/min})_{\text{Calibrator}}$: Rate of change per minute for α-amylase Color Calibrator.

The analyses were performed in triplicate.

Quantitative protein determination

The plasma total protein was estimated by the method described by Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Determination of liver glycogen content

The ortho-toluidine method (Sasaki et al. 1972) was used to evaluate the glycogen content in liver tissues. 500 mg of liver was incubated for 30 min at 100 °C in the presence of 1 ml of 30% potassium hydroxide solution. Then, absolute ethanol (1.5 ml) was added to each tube and mixtures were centrifuged at 2400 rpm for 20 min. The glycogen pellets were then dissolved in 1.25 ml of distilled water and hydrolyzed with 0.25 ml of 30 M HCl for 2 h at 100 °C. Glucose obtained from glycogen hydrolysis was then determined using the o-toluidine reagent and the absorbance was read at 630 nm.

Measurements of oxidative stress parameters in the liver and the kidneys

Lipid peroxidation assay

Concentrations of the malondialdehyde (MDA) in tissues, an index of lipid peroxidation and oxidative stress, was determined using thiobarbituric acid-reactive substances (TBARS) assay, referring to the method of Yagi (1976). The absorbance was measured at 530 nm and the results were expressed as the amount of MDA (nmol) per mg of protein.

Evaluation of the activities of antioxidant enzymes

The method of Sun et al. (1988) was used to determine SOD activity. Enzymatic activity is directly proportional to the inhibition rate of nitroblue tetrazolium (NBT) oxidation by O_2^- anion. The absorbance was read at 580 nm and the activity was expressed as U/mg protein in liver and kidneys.

The method of Flohe and Gunzler (1984) was employed to determine the GPx activity. GPx induced the oxidation of glutathione (GSH), coupled to the transformation of DTNB (5,5-dithiobis-(2-nitrobenzoic)-acid) into TNB (2-nitro-5-thiobenzoate). The absorbance was measured at 412 nm and results were expressed as U/g protein in liver and kidneys.

The Aebi method (Aebi 1984) was adopted to measure CAT activity. The enzymatic reaction was initiated by adding 20 µl of 500 mM H_2O_2 to the supernatant. The absorbance of H_2O_2 decomposition rate was measured at 240 nm and the catalase activity was expressed as U/mg protein in liver and kidneys.

Determination of glutathione levels

GSH levels were determined using the Ellman's reagent (Ellman 1959). A volume of 500 µl of supernatant from tissue homogenate was mixed with 2 ml of 5% trichloroacetic acid. The mixture was then centrifuged at 2000 rpm for 15 min and 500 µl of supernatant were added to Ellman's reagent in 100 mM phosphate buffer (pH 8). After 10 min, the absorbance was measured at 412 nm and the amount of GSH was expressed as mmol/mg protein. A standard graph was plotted using different concentrations of a standard GSH solution.

Hepatotoxicity analysis

Hepatic enzymes, asparate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were used as the biochemical markers of the hepatic damage. The serum enzymatic activities of AST, ALT and ALP were determined using commercial kits (Biomaghreb, Tunisia) on an automatic biochemistry analyzer in the Hospital Hedi Chaker of Sfax, Tunisia. The enzyme activities were expressed as U/ml.

Kidney function tests

Serum creatinine, uric acid and urea concentrations were carried out using commercial kits (Biomaghreb, Tunis, Tunisia) on an automatic biochemistry analyzer in the Hospital Hedi Chaker of Sfax, Tunisia.

Histopathological examination

Pieces of liver and kidneys from the different groups of rats were fixed in a Bouin solution for 24 h and then transported in a 10% formalin solution. The fixed tissues were embedded in paraffin and sectioned at 4-µm thick. Sections were then stained with hematoxylin-eosin and examined under a Motic AE2000 light microscope.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM) and analyzed using the Statistical with SPSS ver. 17.0, professional edition. A one-way analysis of variance (ANOVA) was then performed and followed by Duncun's test to estimate the significance among the main effects at the 5% probability level. Significant differences ($p<0.05$) between means were identified by multiple comparisons across the six groups using least significant difference (LSD) procedures.

Results and discussion

Preparation of fermented sardinelle protein hydrolysates

Many variables greatly influenced the nature of biopeptides produced (the molecular weight and amino acids composition and sequences of protein hydrolysates), and thus their biological and functional properties, including protease specificity. In this study, two protein hydrolysates were obtained by fermentation of sardinelle proteins using *B. subtilis and B. amyloliquefaciens* producing different proteolytic enzymes. After 24h of fermentation, An6 strain showed higher hydrolytic activity (DH=24.3%) than A26 (DH= 21.56%). The differences in DH values are essentially due to the difference in the specificity of enzymes produced by the proteolytic bacteria used. The high DH obtained could be due to the synergistic effects of the multiple proteases produced by the strains during fermentation.

However, these DH are lower than those of fermented Pacific whiting fish sauce (40%) (Tungkawachara et al. 2003) and fermented anchovy by-products sauce (50%) (Yu et al. 2014)

Characterization of FSPHs

The chemical composition of freeze dried FSPHs was determined and compared to that of the undigested sardinelle proteins. The proximate composition of dried USP showed that it had higher protein content (76.43% of dry matter basis) than that contained in FSPHs (75%) ($p<0.05$). The fat content in the hydrolysates was about 0.07%, which is lower than that of USP (5.44%) ($p<0.05$). The low lipid content allows a high stability of hydrolysates towards lipid oxidation. FSPH-A26 and FSPH-An6 had high ash content, 9.8% and 9%, respectively ($p<0.05$).

The amino acid compositions of FSPHs and USP, expressed as residue per 100 amino acid residues, are reported in Table 1. The amino acid compositions of the different samples revealed that they are rich in Asx, Glx, Lys and Arg. Gly was mainly detected in USP (10.62%), while it was found in lower levels in FSPH-A26 (5.77%) and FSPH-An6 (6.05%). Taurine, a sulfur-containing amino acid derived from methionine and cysteine, was also found in the undigested muscle and FSPHs in low quantities. Bouckenooghe et al. (2006) reported that Taurine may be used as a nutritional supplement in functional foods to protect against oxidative stress, neurodegenerative diseases or atherosclerosis. However, Cys and Trp were not detected because they were destroyed under the experimental conditions.

The total of essential amino acids in USP (36.35%) was lower than those of FSPH-A26 and FSPH-An6 (45.38% and 39.52%, respectively). The total of EAA of FSPHs was higher than that of thornback ray muscle hydrolysates (31.9 to 34.1%) (Lassoued et al. 2015). The

presence of interesting amount of essential amino acids in FSPHs justifies their high nutritional value and thus they could be added in functional food formulations.

Effect of FSPHs on hematological parameters

The hematological parameters of the different groups are reported in Table 2. Data showed that there is no significant differences among all the groups ($p>0.05$). The oral administration of rats with 400 mg/kg body weight/day of USP and FSPHs for 10 consecutive weeks didn't induce any change in hematological parameters which proved that there is no pathological risk among the different groups (such as anemia, bleeding disorders, decreased immune defense, mononucleosis, etc.). Our findings are in line with those reported by El-Demerdash et al. (2004) who found that the administration of β-carotene, vitamin E and/or their combination did not cause any significant change in hematological parameters.

Hypoglycemic effects of FSPHs

Effects of FSPHs on blood glucose levels

The levels of blood glucose in the experimental rats are shown in Fig. 1A. The findings revealed that, while the control rats showed normal blood glucose level (4 – 6 mmol/l), the HCD rats showed high levels of glycemia. In fact, the level of blood glucose in rats fed HCD increased by 32% when compared to that of the control group, which proves that the adopted diet induce hyperglycemia state. Interestingly, the oral administration of FSPH-A26 and FSPH-An6 to HCD-fed rats decreased plasma glucose levels by 14% and 22%, respectively, compared to HCD group. The reduction of blood glucose levels indicated that FSPHs contain hypoglycemic peptides which could keep their reactivity under *in vivo* conditions. However, undigested proteins were not able to decrease plasma glucose level, indicating the importance of protein hydrolysis through fermentation.

The obtained results are in agreement with several works previously reported in the literature with regards to the hypoglycemic effects of natural peptides and protein hydrolysates. In a recent study, Nasri et al. (2015) reported the hypoglycemic effects of protein hydrolysates from goby on high-fat-high-fructose diet-fed rats.

Effect of FSPHs on glucose tolerance

The oral glucose tolerance test (OGTT) is generally used to evaluate the capacity to reduce blood glucose level after the administration of an excessive glucose dose, in order to detect glucose tolerance states. The incremental changes in plasma glucose concentration of the different rats, at selected time intervals following the oral glucose challenge, are illustrated in Fig. 1Ba. All the curves showed similar profiles. Indeed, the first 30 min following the glucose administration are characterized by a great increase of the glycemia among the different groups; thereafter blood glucose decreased progressively and reached normal levels after 2 h. HCD-fed rats showed the highest serum glucose level after 30 min, which reached 165 mg/dl, whereas, it doesn't exceed 155 mg/dl in the treated groups. Furthermore, during the last 90 min, the reduction rate of blood glucose level in control, HCD-FSPH-A26 and HCD-FSPH-An6 groups was faster than those observed in HCD and HCD-USP groups. Oral administration of FSPHs to HCD-induced hyperglycemic rats showed similar glycemia reestablishment compared with control rats which proved the preventive effect of FSPHs against the prevalence of the primarily symptoms of T2DM. After 120 min, glucose level of HCD rats was restored; may be due to the presence of a pre-diabetic state in these rats.

To better understand the hypoglycemic effect of FSPHs, the AUC (area under curves) of blood glucose level during OGTT was determined. Fig. 1Bb shows that AUC of plasma glucose concentration relative to the HCD group was higher by 10.52% compared to that of control rats. This rise was moderately corrected by the treatment with FSPHs, and the

maximal reduction percentage was observed in FSPH-A26 group. However, glucose tolerance was not improved in USP-treated rats. Because glucose intolerance status is the principal characteristic of T2DM, the present results suggested that the regular administration of FSPHs could prevent from diabetic risks.

Effect of FSPHs on α-amylase activity in plasma

The results illustrated in Fig. 1C show that the α-amylase activity in the serum of rats fed HCD was significantly higher than that of the control group (+ 86%) ($p<0.05$). Interestingly, the oral administration of FSPH-A26 and FSPH-An6 to HCD-fed rats resulted in a significant decrease in α-amylase activity by about 40% and 45%, respectively compared to HCD group and the obtained activity levels were comparable to that of control group. However, administration of USP to HCD-fed rats didn't decrease the level of α-amylase activity. These findings are in agreement with those of Nasri et al. (2015) who reported that goby protein hydrolysates and not undigested goby proteins were found to decrease α-amylase activity. The obtained results proved that administration of FSPHs could protect rats against hyperglycemia induced by hypercaloric diet. The differences recorded between the α-amylase inhibition abilities of the two protein hydrolysates under investigation could presumably be attributed to the differences in the chain lengths and amino acid sequences of their peptide contents.

Hepatic glycogenesis

Hepatic glycogen synthesis and breakdown play an important role in modulating blood glucose levels (Saltiel and Kahn 2001). The results illustrated in Fig. 1D show that the hepatic glycogen level in HCD rats increased by 330% compared to that of the control group ($p<0.05$). Interestingly, oral administration of FSPH-A26 and FSPH-An6 to rats fed HCD resulted in a significant decrease in hepatic glycogen content by 71% and 58.5%, respectively,

compared to HCD group. The greatest decrease of hepatic glycogen level was recorded in FSPH-A26 treated rats. However, treatment with USP resulted in a slight decrease in glycogen content and the level was 3 fold higher than that of the control group.

The present results proved that the increased consumption of fructose rich-diet induce hepatic glycogen accumulation due to the inhibition of glycogen breakdown rather than the enhancement of its synthesis (Youn et al. 1987). Interestingly, FSPHs are capable to modulate the glycogenesis cascade in liver of hyperglycemic rats; this corrective effect may be due to the regulation of enzymatic activities of glycogen synthase and/ or glycogen phosphorylase in order to ameliorate the hepatic glycogen deposition. Similar findings proved that the treatment with Rosiglitazone posses a preventive effect against hyperglycemia and glucose intolerance risks by modulating hepatic glucose metabolism in high fructose-diet model rats (Yadav et al. 2009).

The overall results (blood glucose levels, glucose tolerance, α-amylase activity and hepatic glycogen levels) proved the ability of FSPHs to attenuate hyperglycemia in HCD-fed rats, unlike USP which did not show any hypoglycemic activity.

Evaluation of antioxidative stress state

Effect of FSPHs on MDA levels in hepatic and renal tissues

It has been established that hyperglycemia leads to increase production of oxygen free radicals (Yadav et al. 2000), which exert their cytotoxic effect by causing lipid peroxidation, resulting in the formation of TBARS. MDA, an endproduct of lipid peroxidation, is widely used as a marker for the occurrence of oxidative stress. The levels of MDA in the liver and kidneys in the experimental rats are shown in Table 3A. An increase in MDA levels by 23% and 24% ($p<0.05$) was observed in the liver and kidneys, respectively, of rats fed HCD,

compared with those fed normal diet. This indicated that HCD induced oxidative stress in the liver and kidneys.

Interestingly, the USP and FSPHs treated rats showed a significant reduction in MDA levels in liver and kidney tissues, and the obtained values were similar and even lower than those of the control group. In fact, oral administration of USP, FSPH-A26 and FSPH-An6 to rats fed HCD decreased the levels of hepatic MDA by 25.00, 43.75 and 68.75%, respectively compared to the HCD group ($p<0.05$). These results suggest that treatment with FSPHs attenuates lipid peroxidation in tissues, and FSPH-An6 was found to be more efficient than FSPH-A26 in the reduction of MDA levels in both tissues.

Antioxidant enzyme activities in the liver and kidneys

In this study, the effect of administration of USP and their hydrolysates to HCD-fed rats on antioxidant enzyme activities (SOD, GPx, CAT) were determined. As reported in Table 3B, SOD, GPx and CAT activities in the liver and kidneys of rats fed HCD decreased significantly as compared to those of the control group ($p<0.05$). Interestingly, the activities of SOD, GPx and CAT, were significantly restored in treated rats, especially those receiving FSPHs. In fact, hepatic and renal SOD activities were significantly higher in HCD-FSPHs groups, as compared to HCD one ($p<0.05$), which increased, by 10.5% (liver) and 35% (kidney) after the treatment with FSPH-A26, whereas FSPH-An6 induced an increase of 51.6% and 42% for hepatic and renal SOD activities. However, undigested proteins exhibited lower antioxidant effect than their hydrolystaes. Indeed, SOD activities increased only by 3.4 % in the liver and by 6 % in kidneys.

In addition, the daily gavage of USP and FSPHs has corrected GPx activities in treated groups. The maximal improvement percentage was observed in FSPH-An6 group (+ 32% in the liver and + 84.5% in kidneys, compared to HCD one) ($p<0.05$). These results were even

higher than the control group. Similarly, in the liver, the CAT activities were improved following the treatment with FSPH-A26 (+28%) and FSPH-An6 (+41%). A better amelioration was observed in kidneys witnessed by the significant rise of CAT enzymatic activities by 62% and 52% after the gavage of 400 mg/kg body weight of FSPH-A26 and FSPH-An6, respectively. This is in agreement with our previous results (Jemil et al. 2016b) showing that FSPH-A26 and FSPH-An6 contained a huge number antioxidant peptides.

Our results are in agreement with those of You et al. (2011) which showed that peptides from loach (*Misgurnus anguillicaudatus*) prepared by papain digestion reduce oxidative stress *in vivo* by increasing the SOD, CAT and GPx activities. Another study reported that the supplementation of the sardinelle muscle protein hydrolysates produced by enzymatic hydrolysis with cholesterol-enriched diet reduces oxidative stress *in vivo* and increases the SOD, CAT and GPx activities (Ben Khaled et al. 2012). These findings were attributed to the presence of small peptides in the hydrolysate mixture that prevent the generation of free radicals.

The over-expression of these antioxidant enzymes in HCD-fed rats treated with FSPHs implies that the native antioxidant defense is probably reactivated by peptides resulting in the increase in the capacity of detoxification through the reactive oxygen radicals scavenging effect.

Glutathione levels in liver and kidney

GSH is a non-enzymatic antioxidant that naturally exists in cells and helps to prevent from cellular damage caused by free radicals and pro-oxidants. The levels of GSH, in the liver and kidneys are shown in Table 3B. Hepatic and renal GSH levels of HCD group were significantly reduced by 19% and 17%, respectively, compared to those of the control group, suggesting that HCD activated the formation of free radicals in the liver and kidneys. This

448 reduction was enhanced by the oral treatment with USP and FSPHs. The effect was more
449 prominent with FSPH-An6. Indeed, hepatic GSH levels increased by 5.26%, 20.5% and
450 29.8%, in USP, HCD-FSPH-A26 and HCD-FSPH-An6 groups, respectively, as compared
451 with that of HCD rats. Furthermore, USP, FSPH-A26 and FSPH-An6 administration
452 increased the renal GSH levels by 23%, 34.07% and 40.26%, respectively, compared to HCD
453 rats. The renal GSH values were even higher than those of the control group.

454 These results may reflect the ability of the hydrolysates to enhance the scavenging and
455 inactivation of H_2O_2 and hydroxyl radicals and thereby terminate the lipid peroxidation chain
456 reaction. This protective effect may be due to the induction of enzymatic and non-enzymatic
457 antioxidants, such as GSH, SOD and CAT in treated animals. Accordingly, the protection
458 afforded by fermented sardinelle hydrolysates against hypercaloric diet-induced ROS (e.g.
459 H_2O_2) generation is likely to be attributable to its antioxidant effects.

460

461 **Effect of FSPHs on hepatic function**

462 AST, ALT and ALP are reliable markers of liver function. The increase in the levels of these
463 markers in plasma was considered as an indicative of active liver damage. As shown in Table
464 4A, there is no significant difference in the activities of AST and ALT in the different
465 experimental groups ($p>0.05$) which indicates the absence of any hepatic inflammation in
466 untreated and treated HCD-fed rats. However, HCD consumption caused a significant
467 increase in the level of ALP (+24%), in comparison to that of the control rats, indicating liver
468 damage. Interestingly, treatment of rats fed HCD with USP, FSPH-A26 and particularly,
469 FSPH-An6 restored the ALP levels. These findings are in agreement with the results
470 previously reported by Ben Khaled et al. (2012) who reported the potential hepatoprotective
471 effect of sardinelle muscle hydrolysates (*Sardinella aurita*). In the same context, wheat gluten

peptides have shown their protective effect against D-galactosamine induced a cute hepatits in rats (Sato et al. 2013).

Effect of USP and FSPHs on kidney function

Creatinine and uric acid are metabolic waste products that are naturally removed from the blood by kidneys via the glomular filtration. Thus, the accumulation of their levels in the bloos is exclusively caused by a renal failure problem. Therefore, kidney indices of toxicity and serum total protein were also investigated (Table 4B). Results showed that in HCD group, a significant increase (+78.43%, $p < 0.05$) in creatinine and a significant decrease (-36.46%, $p < 0.05$) in uric acid were observed compared to control rats. However, the oral administration of USP and FSPHs to HCD fed rats decreased significantly the creatinine levels by 43.59, 44.73 and 41.16%, respectively ($p<0.05$), as compared to that of the HCD group. In addition, uric acid values have been restored and became similar to those of control group. However, no significant difference in urea levels in serum is observed in the different groups of rats.

The total protein content in HCD rats was significantly lower than that observed in the control group (7.72±0.45 vs 6.3±0.65 mg/dl). Reduction in serum total protein observed in hyperglycemic rats may be due to a decrease in protein synthesis, an increase in protein catabolism, a microproteinuria and/or albuminuria, which are important markers of diabetic nephropathy (Mauer et al. 1981). Interestingly, oral administration of FSPH-An6 was found to restore protein content; while USP and FSPH-A26 were found to increase slightly protein content and values obtained were still lower than those of the control group.

Similar findings were observed in previous studies that proved the renal protective effect of fish protein hydrolysates on diabetic rats (Ktari et al. 2014). In another study, Nasri et al. (2015) have shown the preventive role of goby protein hydrolysates against kidney deterioration and uric acid hyperfiltration.

Histological examination

In order to assess the effect of FSPHs on ameliorating the hepatic function, histological analysis of liver tissues of the different rats groups was investigated (Fig. 2A). Microscopic observation revealed the presence of observable lipid vacuoles in the hepatocytes of HCD group as indicated by the arrows in the figure (Fig. 2Ab). However, compared to the hypercaloric-fed rats, except USP group, in which small clear lipid droplets were observed (Fig 2Ae), these vacuoles were absent in the normal rats (Fig. 2Aa), as well as FSPHs-treated animals. The formation of lipid droplets in hepatocytes is likely associated to the hypercaloric diet rich in fat and fructose. Furthermore, the liver architectures of the different group had a normal appearance, which indicated the absence of neither inflammatory or toxicity states in the treated animals. This observation is in line with the biochemical analysis dealing with AST, ALT and ALP activities.

Histological study of the normal kidney of the control rats revealed normal glomerulus surrounded by the Bowman's capsule (Fig. 2Ba). The kidneys of HCD-fed rats (Fig. 2Bb) showed glomerular atrophia and an increase in glomerular space which reflected the enhancement of glomerular filtration rate in these rats leading to the accumulation of toxic products (especially creatinine) in plasma (Mude et al. 2012). However, the groups that were treated with USP and FSPHs showed normal glomerulus and glomerular space which appeared similar to that of control group. Similar findings were found with protein hydrolysates from goby (Nasri et al. 2015).

Conclusion

The present study indicated that HCD induced hyperglycemia associated with an increase of oxidative stress. Administration of FSPHs was found to attenuate hyperglycemia. They also

protected the liver and kidney functions from various injuries caused by hyperglycemia complications. Overall, these results indicated that FSPHs could open new promising opportunities for the production of efficient, safe, and cost-effective natural bioactive peptides. Further studies are needed to purify and identify hypoglycemic and antioxidant peptides from FSPHs. This could provide a versatile supply of beneficial sardinelle proteins that can be incorporated as supplements in health-care food and/or complements to other hypoglycemic foods used to alleviate and/or prevent the long-term complications of type 2 diabetes mellitus.

Acknowledgements

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694 **Table 1**

695 Amino acid composition of undigested sardinelle proteins and its fermented hydrolysates.

	Residue/100 amino acis residues		
	USP	**FSPH-A26**	**FSPH-An6**
Aspartic acid (Asx)[1]	8.80 ± 0.11 [b]	11.72 ± 0.19 [a]	11.67 ± 0.34 [a]
Glutamic acid (Glx)[1]	13.50 ± 0.11 [c]	13.90 ± 0.22 [b]	16.71 ± 0.07 [a]
Serine (Ser)	2.92 ± 0.11 [b]	2.64 ± 0.05 [c]	3.54 ± 0.03 [a]
Histidine (His)[2]	4.65 ± 0.19 [a]	1.36 ± 0.14 [b]	1.31 ± 0.01 [b]
Glycine (Gly)	10.62 ± 0.11 [a]	5.77 ± 0.03 [c]	6.05 ± 0.41 [b]
Threonine (Thr)[2]	4.97 ± 0.08 [b]	5.91 ± 0.09 [a]	4.95 ± 0.51 [b]
Alanine (Ala)	8.30 ± 0.27 [a]	3.91 ± 0.15 [c]	5.92 ± 0.02 [b]
Arginine (Arg)	6.51 ± 0.40 [c]	7.14 ± 0.21 [b]	7.33 ± 0.01 [a]
Taurine (Tau)	1.03 ± 0.07 [b]	0.37 ± 0.01 [c]	1.76 ± 0.13 [a]
Tyrosine (Tyr)	1.13 ± 0.20 [c]	3.54 ± 0.16 [a]	2.34 ± 0.10 [b]
Valine (Val)[2]	2.56 ± 0.07 [c]	4.04 ± 0.02 [a]	3.40 ± 0.31 [b]
Methionine (Met)[2]	1.54 ± 0.04 [c]	3.15 ± 0.04 [a]	2.31 ± 0.07 [b]
Phenylalanine (Phe)[2]	2.30 ± 0.19 [c]	4.08 ± 0.11 [a]	2.81 ± 0.07 [b]
Isoleucine (Ile)[2]	1.80 ± 0.04 [c]	2.41 ± 0.04 [a]	2.31 ± 0.14 [b]
Leucine (Leu)[2]	3.54 ± 0.13 [b]	5.07 ± 0.06 [a]	5.03 ± 0.06 [a]
Lysine (Lys)[2]	14.99 ± 0.03 [c]	19.36 ± 0.13 [a]	17.40 ± 0.51 [b]
Hydroxyproline (Hyp)	5.00 ± 0.01 [a]	0.70 ± 0.01 [c]	1.15 ± 0.10 [b]
Proline (Pro)	5.84 ± 0.06 [a]	4.84 ± 0.07 [b]	4.02 ± 0.26 [c]
EAA	36.35	45.38	39.52
Total	100	100	100

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697 USP, FSPH-A26 and FSPH-An6 represent undigested sardinelle proteins, fermented sardinelle protein
698 hydrolysate obtained by *B. subtilis* A26 and fermented sardinelle protein hydrolysate obtained by *B.*
699 *amyloliquefaciens* An6, respectively.
700 Values are given as mean ± SD from duplicate determinations (n= 2).
701 [a,b,c] Different letters in the same line indicate significant differences ($p < 0.05$).
702 [1] Asx = Asp + Asn; Glx= Glu + Gln.
703 [2] Essential amino acids.

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Table 2

Hematological parameters of control and experimental groups of rats.

	CD	HCD	HCD+ USP	HCD+FSPH-A26	HCD+FSPH-An6
WBC (10^3 / µl)	18.23 ± 1.04	19.57 ± 1.10	17.60 ± 1.40	17.04 ± 2.11	17.25 ± 1.77
RBC (10^6 / µl)	9.23 ± 0.27	9.09 ± 0.54	8.82 ± 0.10	8.56 ± 0.06	8.55 ± 0,21
Hgb (g/dl)	14.83 ± 0.45	13.40 ± 0.15	13.94 ± 0.08	14.00 ± 0.34	13.73 ± 0.12
Htc (%)	47.18 ± 1.35	45.70 ± 2.88	44.40 ± 0.31	43.08 ± 0.96	42.96 ± 0.37
MCV (fL)	51.34 ± 0.38	50.28 ± 0.17	50.37 ± 0.50	50.95 ± 0.53	50.05 ± 0.50
MCH (pg)	16.12 ± 0.12	15.70 ± 0.07	15.72 ± 0.15	16.28 ± 0.30	15.73 ± 0.24
MCHC (g/dl)	31.40 ± 0.14	31.25 ± 0.17	31.23 ± 0.13	31.74± 0.15	31.45 ± 0.25
Platelets (10^3/µl)	925.60 ± 85.29	1073.25 ± 96.94	1021± 18.59	974.5 ± 27.20	1060.17± 55.03
Lymphocytes (%)	73.74 ± 2.76	64.95 ± 4.41	67.98 ± 3.91	70.03 ± 3.20	70.57 ± 3.44

CD : rats fed a control diet ; HCD : rats fed a hypercaloric diet ; HCD+USP : rats fed a hypercaloric diet and received daily USP ; HCD+FSPH-A26 : rats fed a hypercaloric diet and received daily FSPH-A26 ; HCD+FSPH-An6 : rats fed a hypercaloric diet and received daily FSPH-An6.
USP, FSPH-A26 and FSPH-An6 represent undigested sardinelle proteins, fermented sardinelle protein hydrolysate obtained by *B. subtilis* A26 and fermented sardinelle protein hydrolysate obtained by *B. amyloliquefaciens* An6, respectively.

Table 3

Effect of different treatments on oxidative stress parameters: lipid peroxidation (MDA) (A) and antioxidant enzyme activities (SOD, GPx and CAT) and glutathione (GSH) levels (B) in the liver and kidneys of control and experimental groups.

(A)

	CD	HCD	HCD+USP	HCD+FSPH-A26	HCD+FSPH-An6
MDA in liver (nmol/mg protein)	0.13 ± 0.01	0.16 ± 0.02	0.12 ± 0.01 [b]	0.09 ±0.01[a, b]	0.05±0.01[a, b]
MDA in kidneys (nmol/mg protein)	0.38 ± 0.03	0.47 ± 0.05[a]	0.31 ± 0.01[a, b]	0.25 ±0.01[a, b]	0.20 ±0.01[a, b]

CD : rats fed a control diet ; HCD : rats fed a hypercaloric diet ; HCD+USP : rats fed a hypercaloric diet and received daily USP ; HCD+FSPH-A26 : rats fed a hypercaloric diet and received daily FSPH-A26 ; HCD+FSPH-An6 : rats fed a hypercaloric diet and received daily FSPH-An6.
USP, FSPH-A26 and FSPH-An6 represent undigested sardinelle proteins, fermented sardinelle protein hydrolysate obtained by *B. subtilis* A26 and fermented sardinelle protein hydrolysate obtained by *B. amyloliquefaciens* An6, respectively.
Data are expressed as mean ± SEM (n=6). [a, b] indicate significant differences compared to the values of CD and HCD groups, respectively, at $p<0.05$.

(B)

	CD	HCD	HCD+USP	HCD+FSPH-A26	HCD+FSPH-An6
Liver					
SOD (U/mg P)	9.44 ± 1.13	5.54 ± 0.51 [a]	5.73 ± 0.34[a]	6.12 ± 0.17[b]	8.40 ± 0.82[b]
GPx (U/g P)	86.15 ± 9.66	68.07 ± 5.39 [a]	71.37 ± 2.78 [a]	78.18 ± 2.33	90.11 ± 2.42[b]
CAT (U/mg P)	336.33 ± 27.81	301.93 ± 17.98	320.00 ± 24.23	385.52± 14.38[b]	426.57 ± 21.01[a, b]
GSH (mmoles/mg P)	2.12 ± 0.18	1.71 ± 0.12[a]	1.80 ± 0.13	2.06 ± 0.09	2.22 ± 0.08[b]
Kidneys					
SOD (U/mg P)	12.19 ± 0.72	10.59 ± 0.49 [a]	11.19 ± 0.30	14.33 ±0.18[a, b]	15.00 ± 0.22 [a, b]
GPx (U/g P)	2.46 ± 0.40	1.75 ± 0.13	2.01 ± 0.15	2.61 ± 0.23 [b]	3.23 ±0.26[a, b]
CAT (U/mg P)	61.36 ± 5.95	47.84 ± 4.02 [a]	65.74 ± 3.07 [b]	77.40 ± 3.20 [a, b]	72.75 ± 2.50 [b]
GSH (mmoles/mg P)	2.72 ± 0.28	2.26 ± 0.37	2.78 ± 0.12	3.03 ±0.16[b]	3.17 ± 0.25 [a, b]

CD : rats fed a control diet ; HCD : rats fed a hypercaloric diet ; HCD+USP : rats fed a hypercaloric diet and received daily USP ; HCD+FSPH-A26 : rats fed a hypercaloric diet and received daily FSPH-A26 ; HCD+FSPH-An6 : rats fed a hypercaloric diet and received daily FSPH-An6.

USP, FSPH-A26 and FSPH-An6 represent undigested sardinelle proteins, fermented sardinelle protein hydrolysate obtained by *B. subtilis* A26 and fermented sardinelle protein hydrolysate obtained by *B. amyloliquefaciens* An6, respectively.

Data are expressed as mean ± SEM (n=6). [a, b] indicate significant differences compared to the values of CD and HCD groups, respectively, at $p<0.05$.

Table 4

Liver profile indices (AST, ALT, ALP) (A) and kidney indices of toxicity (creatinine, urea and uric acid) (B) of control and experimental groups of rats.

(A)

	CD	HCD	HCD + USP	HCD + FSPH-A26	HCD + FSPH-An6
AST (U/L)	204.20 ±19.02	203.33 ± 8.82	200.80 ± 20.69	192.20 ± 20.65	190.00 ± 13.54
ALT (U/L)	76.80 ± 3.97	70.50 ± 6.90	72.25 ± 5.31	75.00 ± 3.21	69.80 ± 2.20
ALP (U/L)	329.25 ± 17.93	418.25 ± 55.49	316.20 ± 25.75^{b}	332.00 ± 10.82	312.20 ± 36.09^{b}

CD : rats fed a control diet ; HCD : rats fed a hypercaloric diet ; HCD+USP : rats fed a hypercaloric diet and received daily USP ; HCD+FSPH-A26 : rats fed a hypercaloric diet and received daily FSPH-A26 ; HCD+FSPH-An6 : rats fed a hypercaloric diet and received daily FSPH-An6.
USP, FSPH-A26 and FSPH-An6 represent undigested sardinelle proteins, fermented sardinelle protein hydrolysate obtained by *B. subtilis* A26 and fermented sardinelle protein hydrolysate obtained by *B. amyloliquefaciens* An6, respectively.
Data are expressed as mean ± SEM (n=6). $^{a, b}$ indicate significant differences compared to the values of CD and HCD groups, respectively, at $p<0.05$.

(B)

	CD	HCD	HCD+USP	HCD+FSPH-A26	HCD+FSPH-An6
Total proteins (mg/dl)	7.72 ± 0.45	6.30 ± 0.65^{a}	6.58 ± 0.19	6.86± 0.11	7.51 ± 0.12
Urea (mmol/L)	7.28 ± 0.37	6.86 ± 0.21	6.66 ± 0.21	6.63 ± 0.13	6.70 ± 0.19
Uric acid (µmol/L)	63.94 ± 4.04	40.63 ± 2.63^{a}	56.22 ± 5.62^{b}	56.01 ± 2.52^{b}	54.16 ± 2.42^{b}
Creatinine (µmol/L)	24.66 ± 1.95	44.00 ± 1.15^{a}	24.82 ± 2.31^{b}	24.32 ± 1.03^{b}	25.89 ± 0.99^{b}

CD : rats fed a control diet ; HCD : rats fed a hypercaloric diet ; HCD+USP : rats fed a hypercaloric diet and received daily USP ; HCD+FSPH-A26 : rats fed a hypercaloric diet and received daily FSPH-A26 ; HCD+FSPH-An6 : rats fed a hypercaloric diet and received daily FSPH-An6.
USP, FSPH-A26 and FSPH-An6 represent undigested sardinelle proteins, fermented sardinelle protein hydrolysate obtained by *B. subtilis* A26 and fermented sardinelle protein hydrolysate obtained by *B. amyloliquefaciens* An6, respectively.
Data are expressed as mean ± SEM (n=6). $^{a, b}$ indicate significant differences compared to the values of CD and HCD groups, respectively, at $p<0.05$.

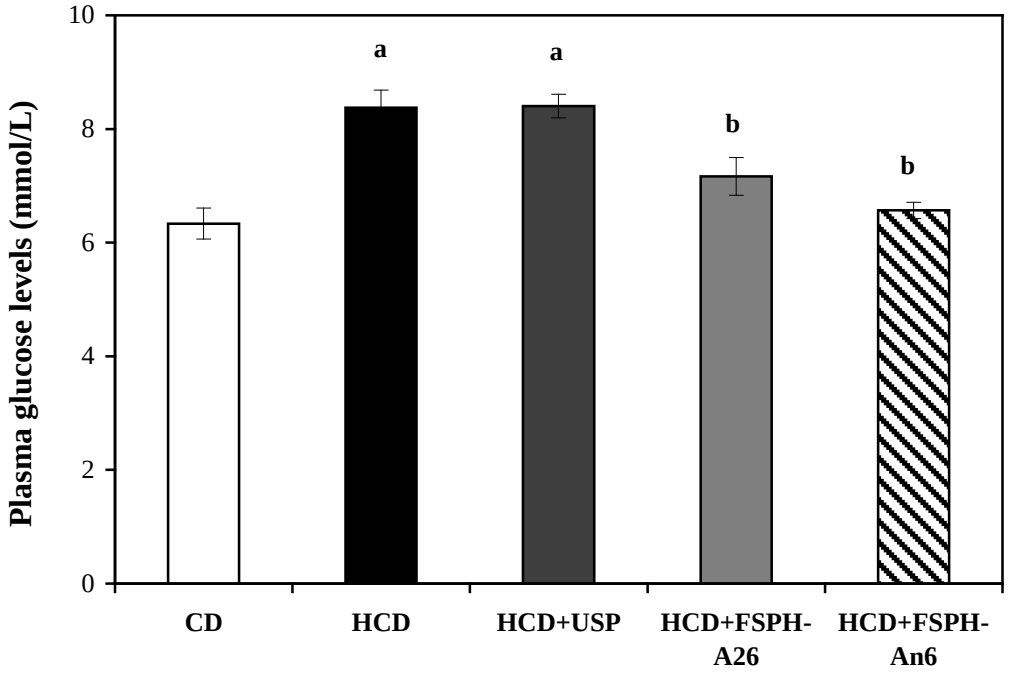
Figures captions

Fig. 1. Blood glucose levels (A), oral glucose tolerance levels (Ba) and their area under curves expressing blood glucose levels as function of time (Bb), levels of α-amylase activity (C) and hepatic glycogen content (D), in control and experimental groups of rats. Data are expressed as mean ± SEM (n=3 for the glucose tolerance test and n=6 for others). [a, b] indicate significant differences compared to the values of CD and HCD groups, respectively, at $p<0.05$.

Fig. 2. Histopathology of liver (×200) (A) and kidney (×200) (B) tissues of control and experimental rats.

Fig. 1.

(A)


Plasma glucose levels (mmol/L)
10
8
6
4
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a
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CD
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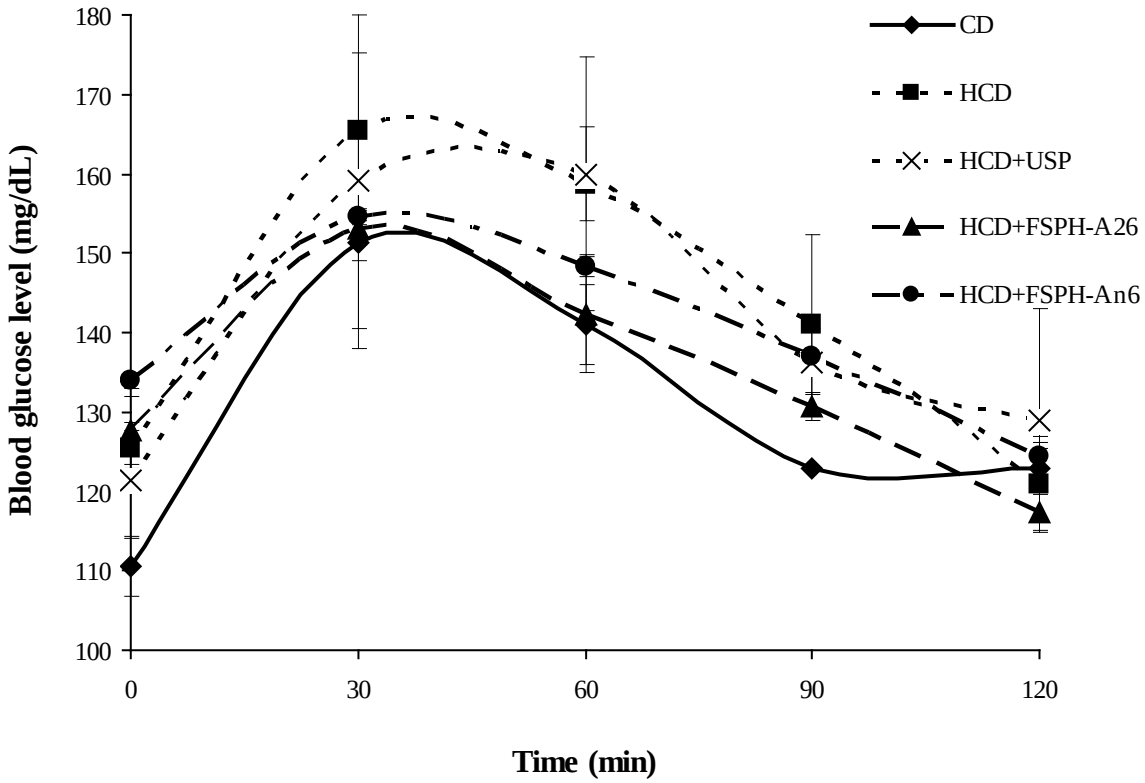
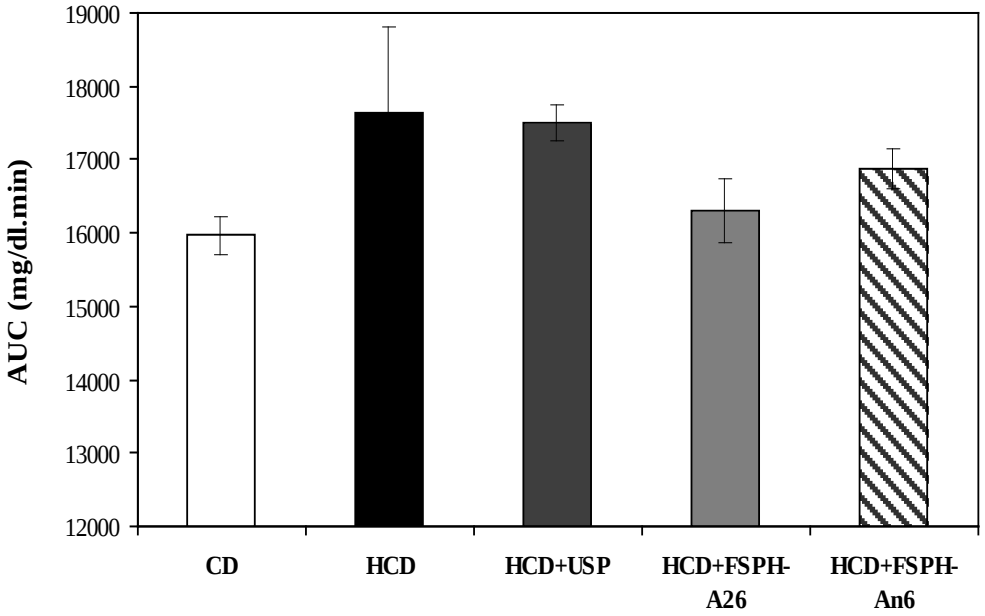
HCD+USP
HCD+FSPH-A26
HCD+FSPH-An6

(a)

(B)


Blood glucose level (mg/dL)
180
170
160
150
140
130
120
110
100
0
30
60
90
120
Time (min)
CD
HCD
HCD+USP
HCD+FSPH-A26
HCD+FSPH-An6

(b)


AUC (mg/dl.min)
19000
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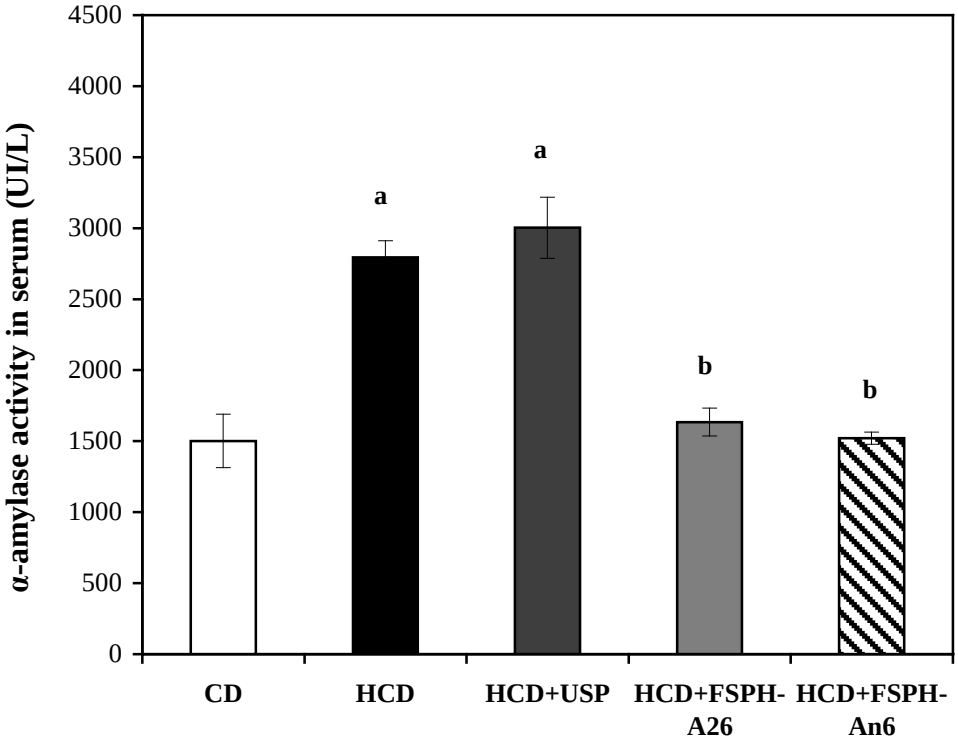
17000
16000
15000
14000
13000
12000
CD
HCD
HCD+USP
HCD+FSPH-A26
HCD+FSPH-An6

(C)


α-amylase activity in serum (UI/L)
4500
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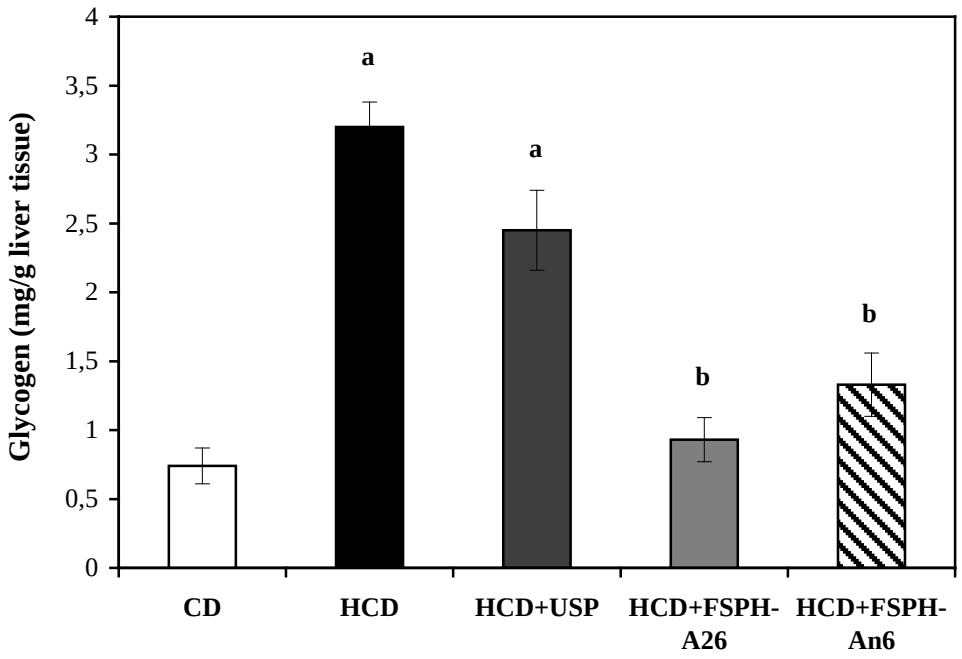
HCD+FSPH-A26
HCD+FSPH-An6

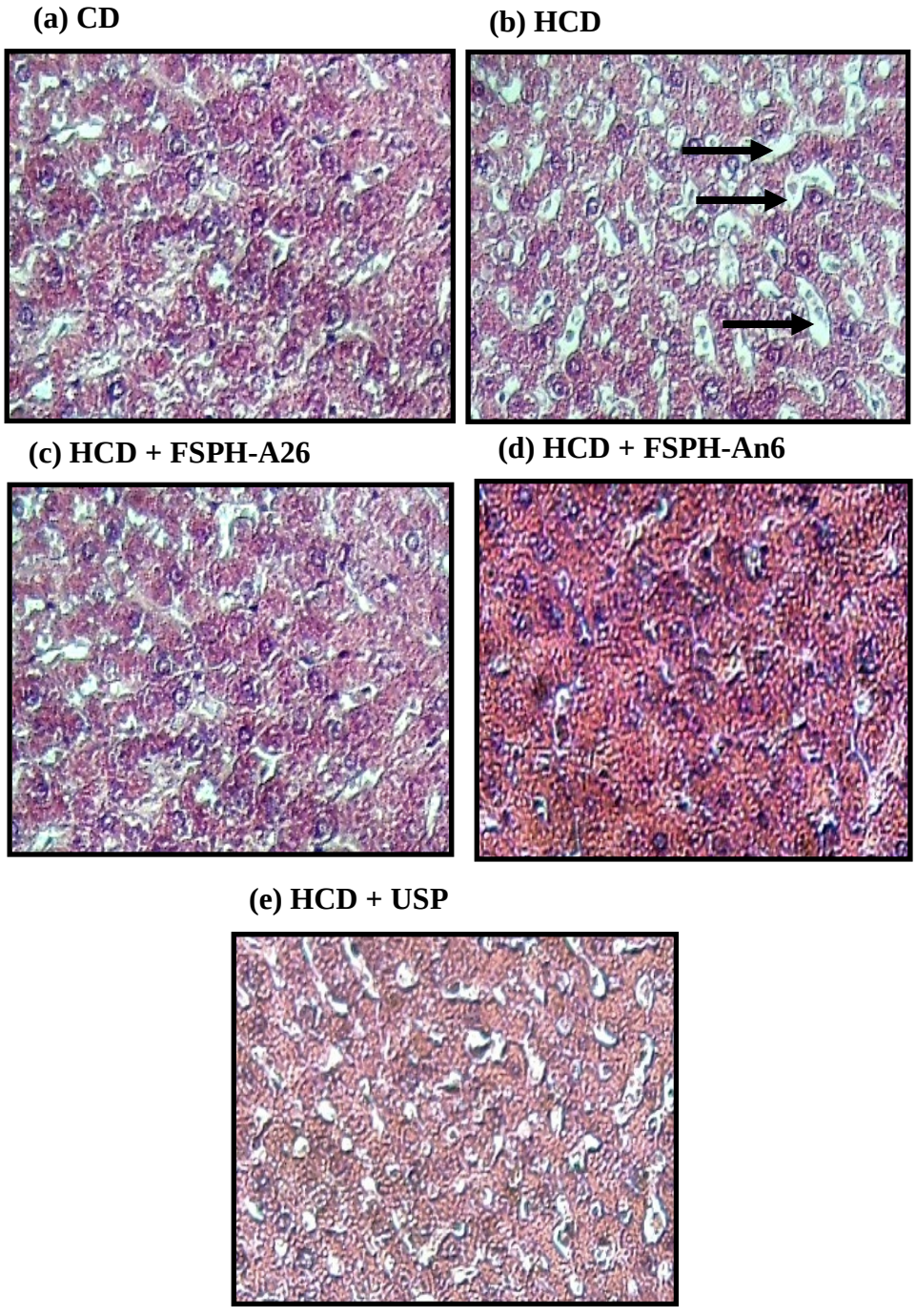
(D)



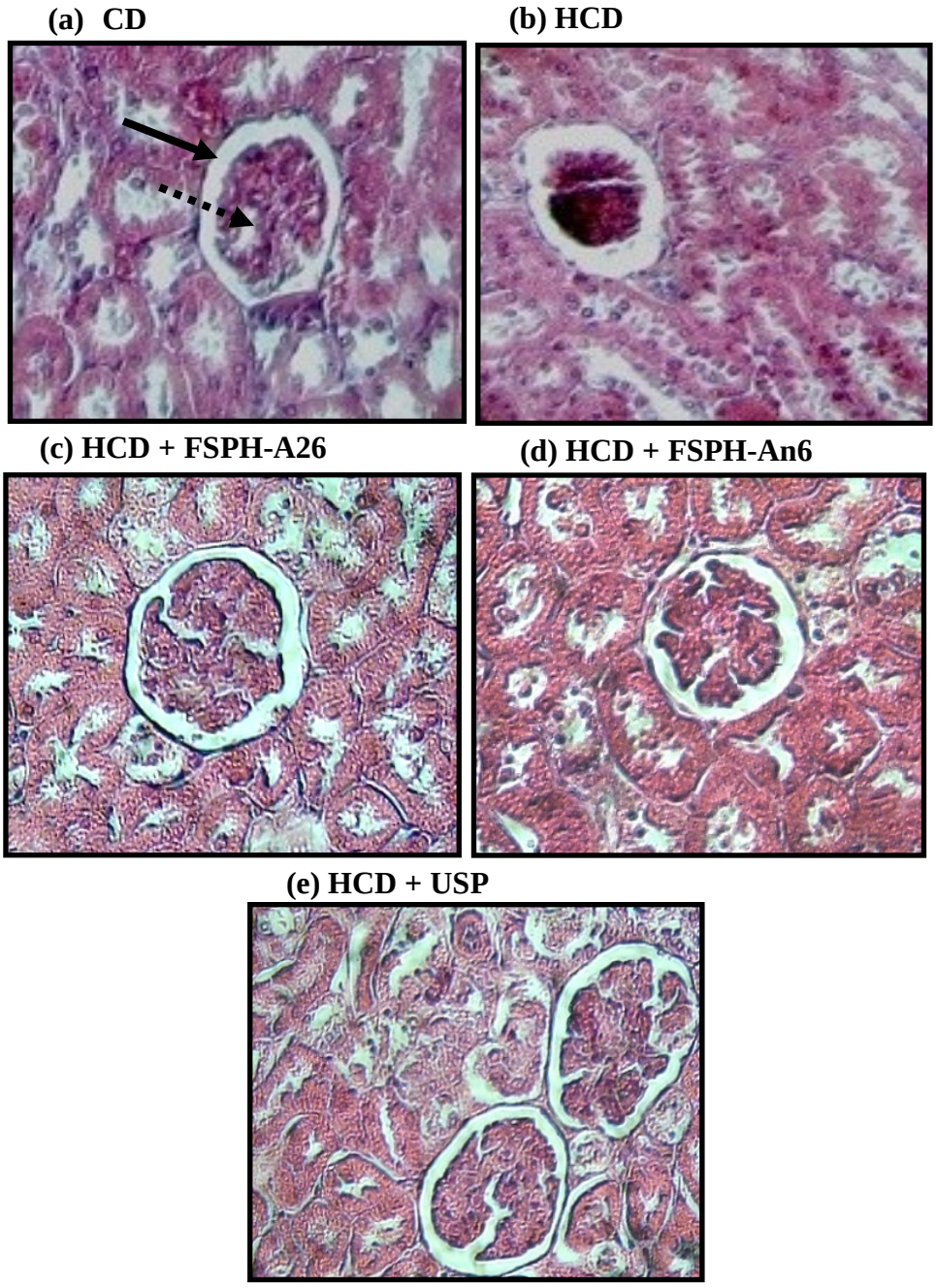
CD : rats fed a control diet ; HCD : rats fed a hypercaloric diet ; HCD+USP : rats fed a hypercaloric diet and received daily USP ; HCD+FSPH-A26 : rats fed a hypercaloric diet and received daily FSPH-A26 ; HCD+FSPH-An6 : rats fed a hypercaloric diet and received daily FSPH-An6.
USP, FSPH-A26 and FSPH-An6 represent undigested sardinelle proteins, fermented sardinelle protein hydrolysate obtained by *B. subtilis* A26 and fermented sardinelle protein hydrolysate obtained by *B. amyloliquefaciens* An6, respectively.

Fig. 2.

A



B



CD : rats fed a control diet ; HCD : rats fed a hypercaloric diet ; HCD+USP : rats fed a hypercaloric diet and received daily USP ; HCD+FSPH-A26 : rats fed a hypercaloric diet and received daily FSPH-A26 ; HCD+FSPH-An6 : rats fed a hypercaloric diet and received daily FSPH-An6.

USP, FSPH-A26 and FSPH-An6 represent undigested sardinelle proteins, fermented sardinelle protein hydrolysate obtained by *B. subtilis* A26 and fermented sardinelle protein hydrolysate obtained by *B. amyloliquefaciens* An6, respectively.