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Research Article

Radical scavenging activity, anti-bacterial and mutagenic effects of Cocoa bean Maillard Reaction products with degree of roasting

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Raw, pre-roasted and roasted Cocoa samples were separated into four different molecular weight fractions (>30, 30–10, 10–5 and <5 kDa) with ultrafiltration and tested for their antibacterial, mutagenic, as well as their radical-scavenging effects. Radical-scavenging effects were tested with electro paramagnetic resonance spectroscopy, anti-mutagenicity in the *Salmonella* microsome assay (with and without metabolic activation), and antibacterial effects by incubating the fractions with several strains of *Bifidobacteria*, *Enterobacter* and *Escherichia,* and observing their growth. The radical-scavenging activity and reducing substance concentrations increased, particularly in the 5–10-kDa roasted fraction. Chromaticity testing elucidated that the 10–5-kDa fraction was one of the darkest fractions. The *Salmonella* microsome assay showed neither mutagenic nor anti-mutagenic effects in any of the samples at any of the different concentrations applied when using TA98, TA100 and TA102. All fractions reduced the growth of pathogenic bacteria, in particular at the highest concentration of 100 μg/mL; however, the same trends were also observed for *Bifidobacteria*.

Keywords: Antibacterial / Cocoa / Maillard reaction products / Radical-scavenging activity / Roasting

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1 Introduction

Cocoa, which is derived from the beans of *Theobroma cacao,* is one foodstuff under investigation due to its high content of initial antioxidants. In particular, polyphenolic constituents such as different catechins or the flavonol quercetin have gained much attention due to their antioxidant capacity (as reducing agents, as free radical scavengers, as metal chelators etc.) and their potential beneficial implications in human health [1]. However, cocoa undergoes several steps of thermal treatment during processing thereby forming Maillard reaction products (MRP), which can be distinguished between early stage "Amadori products" or advanced brown-pigments termed "melanoidins", which all contribute to sensory properties of foods and to functionality such as radical-scavenging potential [2–5]. This has been recently demonstrated in biscuits [6]. Very recent publications also indicated that MRP may have prebiotic activity [7, 8] and may affect the growth of human large-bowel bacteria [9]. In contrast, some reports regarding the safety of different MRP describe their mutagenic and genotoxic potential in various model systems such as glucose-lysine [10], some sugar-casein model systems [11], glucose-glycine [12–14] and very recently in MRP, which have anti-browning activity [15].

The mutagenic/anti-mutagenic activities of heated foods such as coffee, tea, meat or beer have also been described in the literature [16–18], but data on cocoa are limited.

No mutagenic activity was found in instant hot cocoa products in the Ames/*Salmonella* test using TA98, YG1024, and YG1029 with metabolic activation [16]. Cocoa powder was found to be inactive in the Ames assay and the mouse lymphoma assay, in cytogenetic assays

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Abbreviations: **EPR**, electro paramagnetic resonance; **MRP**, Maillard reaction products; **MW**, molecular weight; **Trolox**, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

measuring chromosome breakage and sister chromatid exchange (SCE), and a cell-transformation assay using BALB/c-3T3 cells [17]. Yamagishi *et al*. [18] found some anti-mutagenic activity of cacao liquor polyphenols in the *Salmonella* microsome assay towards the mutagenic action of heterocyclic amines. However, mutagenic activity was found in extracts of instant coffee and roasted coffee beans in the TA100 *in vitro* independent of the variety examined. When considering the influence of the roasting process, green coffee beans showed no mutagenic activity, but mutagenicity increased with roasting time up to 4 min and then remained constant. The genotoxic compounds were quickly formed at temperatures below 220°C. Mutagenic activity was independent of the roasting technology [19]. Extracts of several grain-based coffee-substitute blends and instant coffees were mutagenic in the Ames/*Salmonella* test using TA98, YG1024, and YG1029 with metabolic activation [16]. Similar results were found by Kato *et al*. [20] for brewed and instant coffee, which increased the number of revertants in the TA98 without metabolic activation.

As no data on cocoa at different roasting stages are available, except for one report of our group published recently [3], this work was carried out to evaluate the effect of cocoa roasting on effects, which have been considered in coffee so far in a broader sense on (i) the anti-mutagenic effects of the bulk and cocoa bean fractions in the *Salmonella* microsome assay with and without metabolic activation, (ii) the free radical-scavenging activity and (iii) possible anti-bacteriological effects. Mainly, the effect of roasting of the cocoa beans, in terms of the formation of Maillard reaction by-products was be investigated.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The *Salmonella thyphimurium* strains were obtained from Discovery Partners International (San Diego, CA).

The cocoa beans (Ghana) were kindly supplied by Lindt & Sprüngli SpA in Varese (Italy). The beans came from three different batches and were mixed in the laboratory to ensure a representative sample. The beans had been subjected to two different heat treatments by the supplier, *i.e.* pre-roasting and roasting. First, the whole beans were treated by infrared and radiant heat to dry and loose the shell of the beans but without significantly affecting the nib. In the second part of the process, the separated nibs were subjected to a treatment where the nibs were first pre-roasted at relatively low temperatures (80–90°C for 10 min) and then roasted at higher temperature (130–160°C for 15–20 min).

Ultra-pure water of >18 MO/cm resistivity and total organic carbon of <5 ppb was obtained from a MilliQ 185 system (Millipore, France), fed by pure water from a Millipore Elix 5 system, the MQ grade being used to make up all solutions.

2.2 Samples and sample preparations

The sample preparation was done as described in detail previously [3]. Briefly, the raw Cocoa beans (2 kg) were hand peeled and crushed with a pestle and mortar to obtain small pieces known as nibs. The nibs were cryogenically ground at −198°C to a fine powder in a Sterilmixer II for 1 min at 17 000 rpm. The pre-roasted (1 kg) and roasted (1 kg) cocoa beans were transferred to the grinder directly as they had already been peeled and crushed during processing. The powder was defatted by extraction with petroleum ether and centrifuged using a Beckman Avanti J-20-Rotor JLA 8.1000 at 3000 rpm for 15 min at 4°C. The supernatants were then discarded. Fresh petroleum ether was added and successively centrifuged (three times). The resulting defatted material was air-dried at room temperature (in pre-weighed petri dishes) and then re-weighed. The fat contents were calculated by subtractive final net weights from the original (found to be ∼33%). The defatted bean powder was extracted in hot water using an automatic shaker bath for 20 min at 70°C. The ratio between cocoa powder and water was 1:8. Subsequently, the aqueous solutions were filtered through paper filters (Schleicher & Schuell, ø 240 mm, grade 0858½ and 7–12 μm). The average extraction yields were 17% for roasted, pre-roasted and raw cocoa beans. The filtrate was split into two parts, one for bulk analysis (∼350 mL) and another for ultrafiltration (∼2100 mL). The bulk was frozen and then freeze-dried using a Lyovac GT2 lyophilizer. The ultrafiltration was carried out using an Amicon 8400 Ultrafiltration device (Millipore) and a regulated N_2 supply to create a slight positive pressure in the filtration device. Ice packs were used to cool the filtrate reception beaker.

To obtain four samples with different molecular weight (MW) ranges, three ultrafiltration membranes (Millipore): 30 kDa (PLCC ∅ 76 mm); 10 kDa (PLCC ∅ 76 mm); 5 kDa (PLCC ∅ 76 mm) were used. The filtrate from the first 30-kDa filter was filtered successively through the 10 and the 5 kDa. In this way, four samples with different MW ranges, >30 kDa, 30–10 kDa, 10–5 kDa and <5 kDa were obtained for each type of cocoa: raw, pre-roasted and roasted, respectively. Aliquots of the filtrates were then transferred to aluminium dishes, frozen at −30°C and freeze-dried. The lyophilized samples were placed in plastic containers and stored in a desiccator over silica gel at room temperature. The lyophilized samples were completely dissolved in hot water and then cooled down before free radical-scavenging activity measurements. The relative ratio of single fractions with respect to bulk was then calculated. The data from reducing substances and free radical scav-

enging activity of the fractions were then reported with respect to content of the original material [3].

2.3 Analytical procedures

2.3.1 *Salmonella* microsome assay

For the tests applied, the strains TA98, TA100 and TA102 have been used in order to consider various mutations within the strain, which make them more sensitive to respond to different mutagens. In particular, mutations of the TA102 can be reverted by mutagens, which cause oxidative damage. In addition, the DNA repair proficient strain TA102 detects cross-linking agents. TA98 provides information on frame-shift mutations and TA 100 on base-pair substitutions. The *Salmonella* microsome assay was used with and without a preincubation period. The preincubation assay, which allows closer contact of the strains to the test compounds, which is important for MRP [14], was applied with a preincubation period of 25 min at 37°C as described earlier [21]. Some genotoxic and carcinogenic chemicals such as aromatic amines or polycyclic aromatic hydrocarbons are biologically inactive unless they are metabolized to active forms, or vice versa. Mutagens are active but they are inactivated during metabolization. Since bacteria do not have a cytochrome-based P450 metabolic oxidation system, a rodent metabolic activation system was introduced into the test system [22, 23] The metabolic activation system consisted of a $9000 \times g$ supernatant fraction of a liver homogenate from Aroclor 1254 pretreated rats [S-9 microsomal fraction (S9-mix)], which was delivered to the test system in the presence of NADP and cofactors for NADPH-supported oxidation [24]. The S9-mix consisted of 19.75 mL $_dH_2O$, 25 mL PBS buffer, 0.5 mL $MgCl_2$ (0.85 M), 0.5 mL KCl (1.65 M) and 2 mL NaDP (90.8 mM), 250 µL glucose-6-phosphate (1.08 M) and 2 mL of S9. The solution was vortexed to form a homogenous solution, was kept on ice and used within 1 h. Specific positive controls were used in order to confirm the reversion properties and specificity of each tester strain, and the efficacy of the metabolic activation system. The 2, 4, 7-trinitro-9-fluorenone for TA98 and TA102 strains and sodiumazide for TA100 without metabolic activation and 2-aminofluorene for all strains with metabolic activation were used as positive controls. Due to the solubility limits, the fractions were tested at least at five different concentrations (10% were possible, 5%, 2.5%, 1%, 0.5% and 0.1%). The cocoa fractions (100 µL), then 100 µL of mutagen solution, 500 µL of PBS or S9 mix (4% v/v) and then 100 µL of overnight bacterial culture were added to test tubes. The tubes were incubated for 25 min at 37°C on a rotary shaker. Subsequently, 2 mL of molten top agar was added to every tube, which was then vortexed and poured onto minimum glucose plates. After the agars were solidified, they were incubated for 48 h at 37°C and the *his*$^+$ revertants were counted manually.

Three plates were counted for each concentration of each condition and each test was repeated on another day ($n = 6$).

Prior to the tests, the strains were analyzed for their genetic integrity and spontaneous mutation rate.

2.3.2 Antioxidant activity with electro paramagnetic resonance spectroscopy

The electro paramagnetic resonance (EPR) bench top spectrometer of high sensitivity for spin resonance spectroscopy, MS 200 (Mini Scope 200-Magnettech, Berlin, Germany) was used to investigate the antioxidant activity of the cocoa fractions mentioned above. For the EPR analysis the stable radical Fremy's salt was used as a spin probe.

The EPR conditions were as follows: magnetic field 3360 Gauss; sweep time 50 s; modulation amplitude 460 mG; microwave power 13 dB (corresponding to ~5 mW) and gain 2 exp100.

For radical preparation 2 mM of Fremy's salt was prepared in phosphate buffer pH 7.4. The EPR spectrum of Fremy's salt radical was obtained at 0 min (as a blank, the signal of unreacted radicals means 100%) and after 20 min, by which time the reaction with antioxidant cocoa compounds was complete. The velocity by which the signal decreases determines the antioxidative activity. The decrease in signal intensity is a measure of the antioxidative capacity.

The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (1 mM; 2.50 mg/10 mL methanol) was prepared in methanol: water (1:1). The Trolox solution was diluted 1:2; 1:4; 1:8 with methanol:water (1:1) to have a concentrations range from 0.0625 to 0.25 mM for the calibration curve.

For cocoa sample preparation, 25 mg of freeze-dried sample (a fraction prepared by chromatography or ultrafiltration or sample of the bulk cocoa) was dissolved in a volumetric flask (5 mL) of warm (~40 – 50°C) pure water and mixed in an ultrasonic bath for 1 min. Each sample was diluted 1:5 before analysis. A 100-µL aliquot of sample solution/or Trolox solution was mixed with 100 µL of Fremy's salt in a 1.5-mL Eppendorf tube and 50 µL of this mixture was utilized for the analysis. For each sample measurement three replicates were done.

2.3.3 Antibacterial activity

The following strains were used in the experiments: *Bifidobacterium lactis* Bb-12, *B. bifidum* B 7.1 [25], *Enterobacter cloacae, Escherichia coli* ATCC 8439, *E. coli O157:H*H7 ATCC 43888. Bifidobacteria were cultured anaerobically (in Anaerobe Jar+GasPak System, Oxoid) at 37°C for 48 h in tryptone peptone yeast extract (TPY) broth, and *E. coli* ATCC 8439*, E. cloacae and E. coli* O157:H7 ATCC 43888 were grown in tryptic soya broth (TSB) at 37°C for 24 h. Raw and roasted cocoa fractions have been tested on antibacterial activity with the liquid-medium method and the agar diffusion-well assay. For the liquid-medium method,

2 mL of the melanoidin solutions of each MW was added to 2 mL doubly concentrated TPY liquid medium. By twofold dilution, the following melanoidin concentrations were set: 100, 50, 25 and 12.5 μg/mL. These liquid media were inoculated with 0.1 mL of 48-h culture of bifidobacteria or with 0.1 mL of 24-h culture of enterobacteria. After 48- (in case of bifidobacteria) or 24-h (enterobacteria) incubation, cell concentrations of the samples were determined by agar plating and by measuring absorbance at 660 nm. All tests were performed in triplicate.

For the agar diffusion-well assay, TPY or TSB agar (depending on the applied strain) was inoculated with the strains (10^6 cells/mL) and poured into petri dishes. Following solidification of the agar, small wells ∅ 0.5 cm) were created in the agar plate. Next, 50 μL of the test solutions was pipetted into the wells. The following concentrations of the cocoa fractions were applied: 10, 5, 2, 1, 0.5 and 0.25 mg/mL. Observation of possible inhibition zones occurred after a 48-h incubation at 37°C under anaerobic conditions in case of bifidobacteria, and in a thermostat in case of enterobacteria.

2.3.4 Total reducing substances

The total reducing substances of the cocoa samples (three replicates each test) were determined by the Folin Ciocalteu method as described previously [3].

2.3.5 Chromaticity testing

Chromametric measurements were carried out using a Chromameter CR-410 (Konica Minolta, NJ), calibrated with a white reference plate and adapted for measuring dissolved substrates with a cylindrical sample holder of internal diameter 60 mm and height 35 mm placed at the top of the Minolta measuring head. The sample holder's internal side surface was black, the bottom surface was made of transparent glass, and the measurements were made with a volume of 20 mL. During the measurements, the cylindrical sample holder was covered by the white reference plate. All samples were diluted for measurement with pure water at the final concentration of 25 mg/20 mL and quadruplicate readings were carried out. The L* a* b* color space was devised in 1976 to provide more uniform color differences in relation to visual differences. In this color space, L* indicates lightness and a* and b* are the chromaticity coordinates. The a* and b* indicate color directions: +a* is the red direction, −a* is the green direction, +b* is the yellow direction, and −b* is the blue direction. The center is achromatic; as the a* and b* values increase and the point moves out from the center, the saturation of the color increases.

2.4 Data analysis

All data are expressed as mean ± SD. Different radical-scavenging responses observed for different concentrations and incubation times were analyzed by one-way analysis of variance (ANOVA) using SPSS 12.0 for Windows. Differences were considered significant at a value of $p < 0.05$.

In addition to the statistical evaluation, a "non-statistical" evaluation was considered for the *Salmonella* microsome assay data ("twofold rule"). The total number of revertants per plate is at least twice as high as the number of spontaneous revertants (negative control) per plate.

3 Results and discussion

Cocoa is a roasted food product and unlike coffee has undergone very few investigations for MRP. To gain insight into the molecular weight of the products formed, which have recently shown to be quite different [1, 3, 14, 26], the cocoa mixtures were separated into four fractions by using ultrafiltration with cut-off filters of 30, 10 and 5 kDa.

The different test systems have been chosen, as MRP are discussed in respect to all these effects [2]. However, sometimes the same substance/substance group shows beneficial effects but on the other hand, when using another test system it is detrimental [14, 15]. Therefore, we decided to investigate the same fractions, on presently hot discussed fields in MRP research, thereby also considering the roasting stage and the MW.

3.1 Chromaticity testing

The results of color parameters of cocoa beans are shown in Fig. 1. The L* parameter indicates lightness and a* and b* are the chromaticity coordinates and indicate red (+a*) and yellow (+b*). Since a* values span only a short range (5.16 – 11), including all samples, this parameter is not considered to be significant.

The brown color is due to lower L* and higher b* values. Therefore, the darkest fractions are the >30, 30 – 10 and 10 – 5 kDa for pre-roasted and roasted while L* and b* remain slightly higher in the raw fractions. The lighter fractions are the <5 kDa raw, pre-roasted and roasted with the roasted fraction being significantly ($p < 0.05$) different (lighter) from the pre-roasted and raw ones. These fractions are also more yellow with respect to the other samples.

Nicoli *et al*. [27] observed that there was a tendency for L*, a* and b* values to decrease for roasted samples because of the brown color development. This was, as mentioned above, noted for the >30, 30 – 10 10 – 5 kDa in the pre-roasted and roasted cocoa fractions. As a general observation, it seems that thermal treatment of the cocoa beans does not induce great changes in the a* values of the respective fractions. The use of ultrafiltration tended to purify the samples and to eliminate the brown pigments that remained associated with the higher MW molecules. It is likely, for this reason that the <5-kDa fractions are lightest in color.

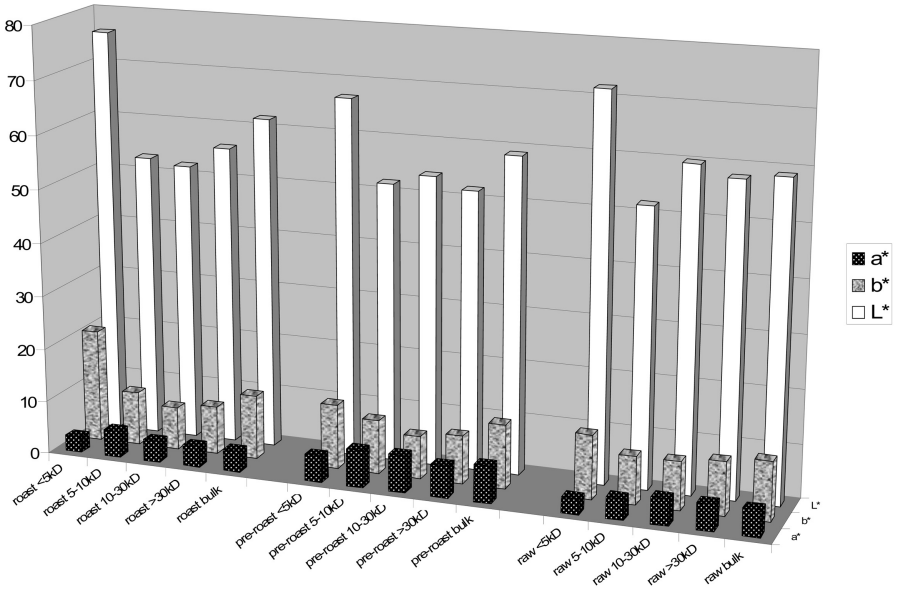

Figure 1. Chromaticity values (a*, b* and L*) of fractions of raw, pre-roasted and roasted cocoa beans. Note: RSD < 1% for all ($n = 3$).

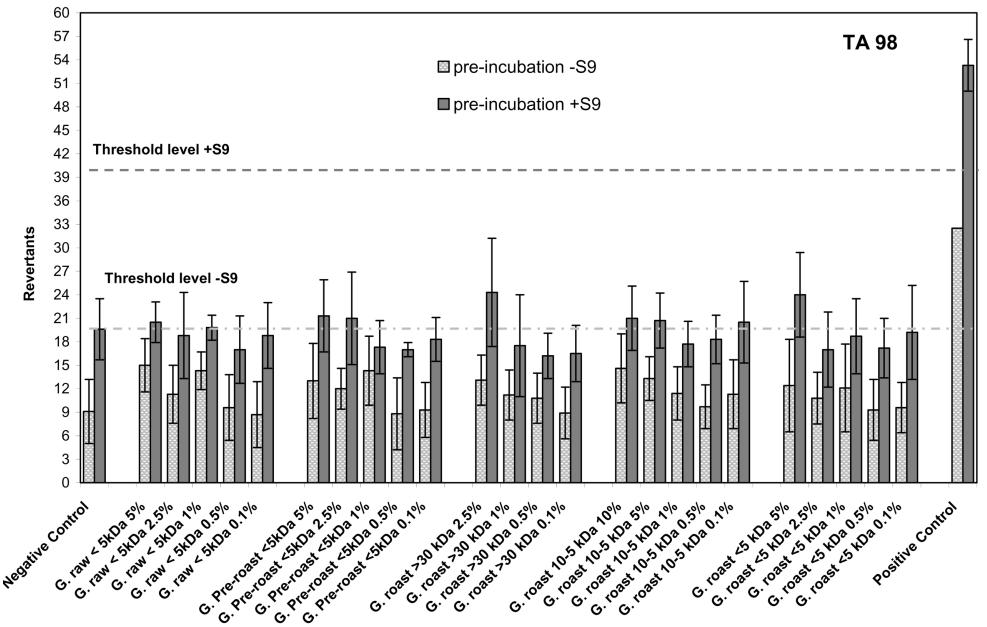

Figure 2. His$^+$ revertants in TA98 induced by water-soluble cocoa fractions of cocoa raw, pre-roasted and roasted (expressed as revertants per plate: mean ± SD). The threshold levels present the number of revertants twice as high as the negative control as sign for mutagenic response.

3.2 Mutagenic activity

Initially, the *Salmonella* microsome assay was applied for the testing without intensive sample preparation. Since the sample preparation in this study could not be carried out under sterile conditions, some of the fractions became contaminated with bacteria. In particular, this was the case for the pre-roasted beans due to an activation of the bacteria by the short heat treatment, which was too short for inactivation. Therefore, the raw > 30-kDa and pre-roasted > 30-kDa fractions could not be taken into consideration for the statistical analyses.

Although several attempts were made to deactivate the bacteria, *e.g.* through irradiation (UV-C, 12 h) or several washing steps with solvents, it was not possible to inhibit colony growth of bacteria other than the *Salmonella*. Although the *Salmonella* showed growth, the samples were not included in the statistical evaluation since it was not sure whether there was an interaction with other bacteria.

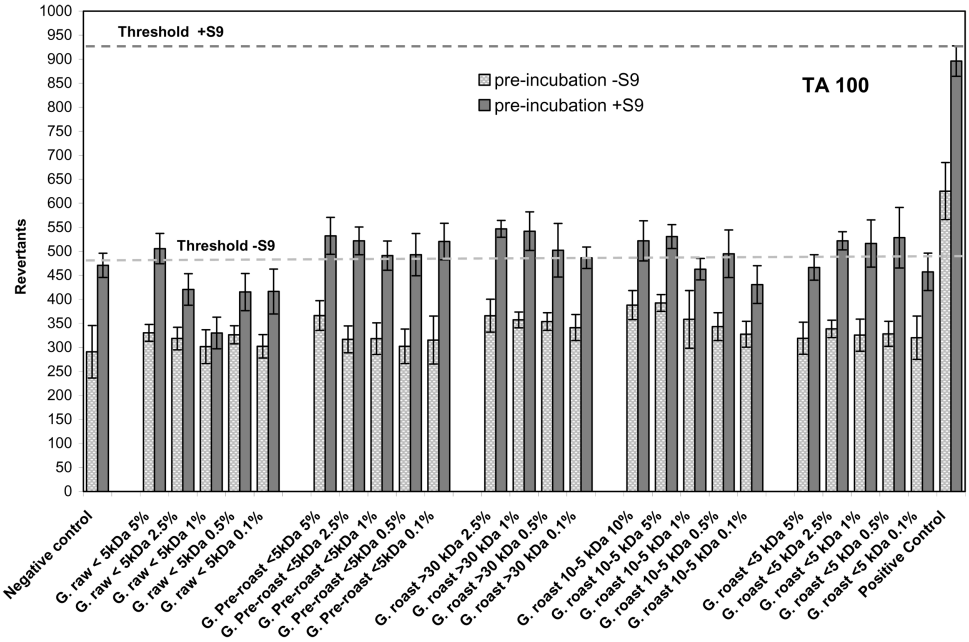

Figure 3. His$^+$ revertants in TA100 induced by water-soluble cocoa fractions of cocoa raw, pre-roasted and roasted (expressed as revertants per plate: mean ± SD). The threshold levels present the number of revertants twice as high as the negative control as sign for mutagenic response.

The spontaneous mutant frequency of the strains was in accordance with the control levels published [23] and remained consistent within the test period.

Figures 2 – 4 show the results of the MW fractions in the *Salmonella* microsome assay in strains TA98 TA100 and TA102, with or without S9 activation and with preincubation. Each tested concentration was significantly lower than the positive control ($p < 0.05$).

In strain TA98 no mutagenic activity, neither without nor with S9 activation was observed. However, there is a non-significant tendency that the highest concentrations of the roasted cocoa beans induced a higher number of revertants than the lower ones, in particular when compared to the concentrations below 1%.

The same observation was true for the TA100.

The stronger response was found in the TA102, which is highly sensitive to oxidative damage. No mutagenic effects were found with the raw and pre-roasted fractions, as no concentration tested exceeded the twofold revertant number of the negative control. Nevertheless, the highest concentrations of the roasted fractions exceeded the threshold level or showed borderline effects. When S9-mix was added (+S9), in order to increase the level of metabolic enzymes, mainly phase-I enzymes, to simulate body conditions, the threshold was not reached. With all roasted fractions, independently of metabolic activation, a concentration-dependent increase ($p < 0.05$) was observed. This increase was stronger ($p < 0.05$) than for the pre-roasted or raw samples. These results prove that in the 102 test the roasting products were able to induce the revertant growth in a concentration-dependent manner, which is a sign of a pro-oxidative effect. Data on the mutagenic effects of roasted cocoa are scanty; the few studies published failed to detect mutagenic properties of cocoa. However, no study compared the effects of raw, pre-roasted and roasted cocoa, in order to consider particularly the roasting effects, which has been shown for coffee [28] or fermented tea [29]. Similarly to the result of Johansson *et al*. [16] we could not find mutagenic effects in cocoa in the TA98 strain, neither without nor with metabolic activation. A higher response was found in TA102 strain, which has not been tested in cocoa so far. The number of revertants increased with the concentration and with the heat treatment. In the roasted samples mainly the lower MW fractions (< 10 kDa) induced the highest colony number, and the increase was concentration dependent, which is an indication of negative effects. Although expected, we were not able to observe anti-mutagenic effects or an effective reduction in revertants in the cocoa fractions.

However, independently of the condition and the strain tested, concentrations below 1%, which are considered more physiologically relevant, were shown to be safe.

3.3 Antioxidant activity

The ability of the cocoa bean fractions to donate a hydrogen atom or electron to the synthetic free radical potassium nitrosodisulfonate (Fremy's salt) monitored by EPR is shown in Fig. 5 expressed as Trolox milliequivalent/mg.

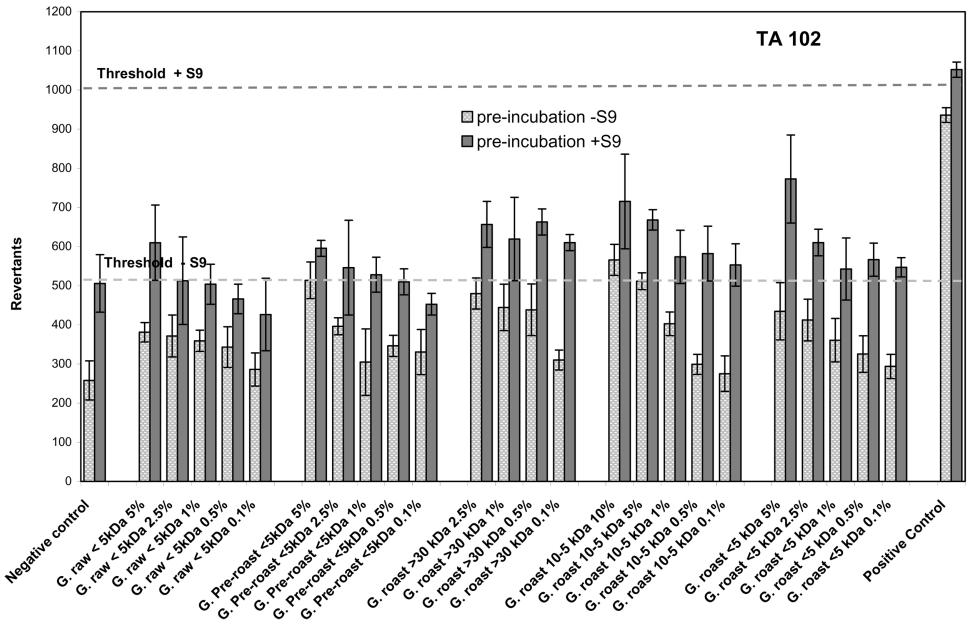

Figure 4. His$^+$ revertants in TA102 induced by water-soluble cocoa fractions of cocoa raw, pre-roasted and roasted (expressed as revertants per plate: mean ± SD). The threshold levels present the number of revertants twice as high as the negative control as sign for mutagenic response.

The radical-scavenging activity in the bulk decreases progressively with pre-roasting and roasting, probably because of the expected degradation of antioxidative active polyphenol compounds, which has been seen in experiments with coffee [30]. However, the most significant changes were observed within the individual fractions, when they were subjected to roasting. The activity in the >30-kDa fraction increased slightly after pre-roasting but decreased significantly ($p < 0.05$) after roasting. In contrast, the activity in the 30–10-kDa fraction decreased slightly after pre-roasting and returned to a similar level as the original raw cocoa after roasting. This could be due to further generation of radical-scavenging substances in the 30–10-kDa fraction as a result of Maillard reaction, possibly occurring due to the high temperature of the roasting process, as it has been shown that at the same fraction the reducing substances increased too [3]. However, in the lower MW polymers, many compounds seem to be incorporated, which are able to form highly active products during the prolonged heating conditions used for roasting [4, 7]. Roasting products such as pyrazines, pyrroles, furans, pyridins or thiazoles, which are well known to be formed in coffee [31, 32] but also in cocoa [33] might also contribute to the antioxidative potential of the roasted samples. This fact is confirmed by the profile found in 10–5-kDa fractions: the roasted samples showed the highest antioxidative potential, which correlated with the concentration of reducing substances ($p < 0.05$; $r^2 = 0.990$; see Table 1) in comparison to raw and pre-roasted fractions ($p < 0.05$). The <5-kDa fraction showed a very low radical-scavenging potential and the lowest concentration of reducing substances. These findings are in agreement with a recent investigation on coffee, which could also prove that the different antihydroxyl radical activity is based on the MW and the roasting process. In particular the high-MW fraction (>3.5 kDa) showed similar activity as the green beans, whereas the lower MW fraction was less effective [34]. The high radical-scavenging potential of the roasted fraction in the 5–10, but also in the 10–30-kDa fractions is therefore due to the presence of reducing substances other than polyphenols, such as higher MW roasting products, which is also supported by the data of the *Salmonella* microsome assay. This is underpinned by the general opinion that the MW range of melanoidins is >5 kDa. This is due to the complexity of the Maillard reaction and the inherent dependence of the MRP on initial sugars, peptides, water activity etc, and roasting conditions. This is supported by the work of Ames [35], who described the wide array of interactions that may occur between melanoidins and flavor compounds.

Table 1. Reducing substances concentrations determined by Folin-Ciocalteu assay in bulk cocoa and individual cocoa fractions (mg/g fraction)

Sample	Raw	Pre-roasted	Roasted
Bulk	124.6 ± 3.1	117.6 ± 2.8	104.5 ± 2.4
>30 kDa	54.4 ± 1.4	68.9 ± 1.8	13.9 ± 0.4
30-10 kDa	52.4 ± 1.3	40.4 ± 1.0	54.2 ± 1.4
10-5 kDa	8.8 ± 0.2	4.5 ± 0.1	32.2 ± 0.7
<5 kDa	8.9 ± 0.3	3.8 ± 0.1	4.2 ± 0.1

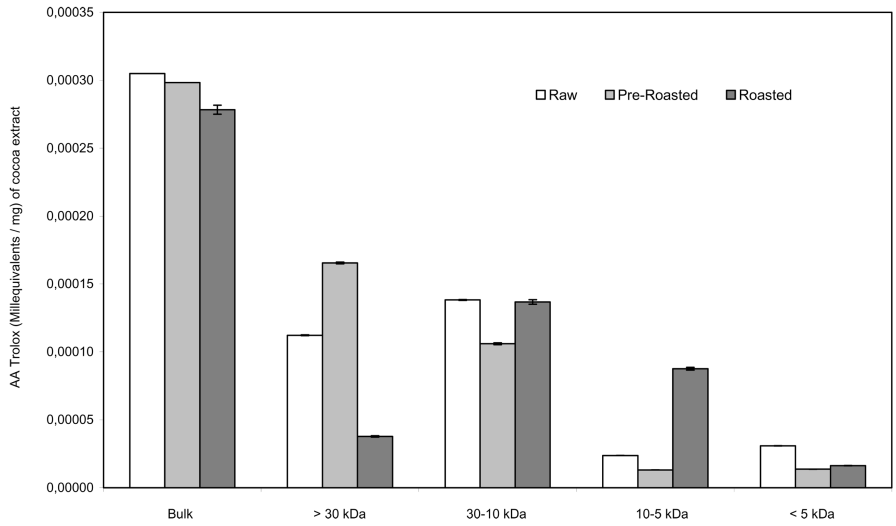

Figure 5. Free radical scavenging activity determined by EPR of bulk cocoa and individual cocoa fractions after 20 min.

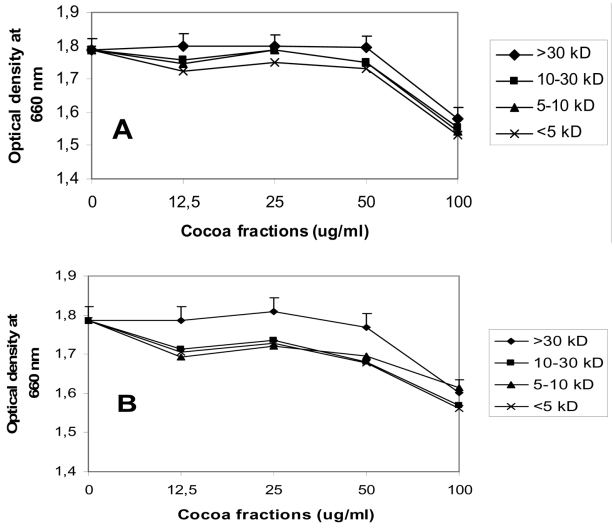

Figure 6. Effects of roasted (A) and non-roasted (B) cocoa fractions on the optical density of *Escherichia coli* ATCC 8439 at 660 nm. SD (Stdv) only shown in one data line, other Stdv similar; 100 μg/mL always < 12.5, 25, 50 ($p < 0.05$).

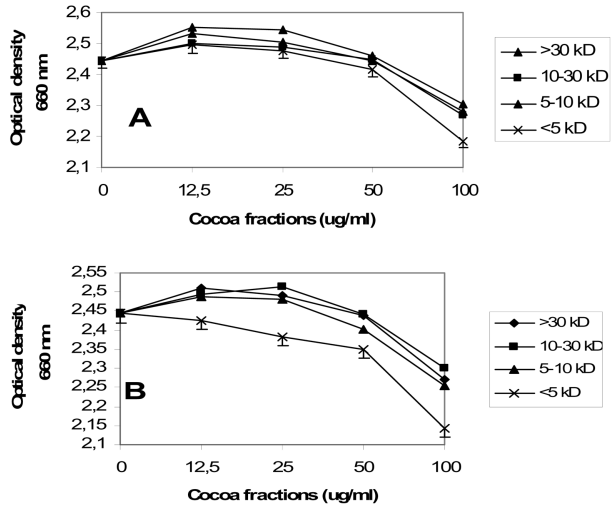

Figure 7. Effects of roasted (A) and non-roasted (B) cocoa fractions on the optical density of *Enterobacter cloacae* at 660 nm. SD (Stdv) only shown in one data line, other Stdv similar; 100 μg/mL always < 12.5, 25, 50 ($p < 0.05$).

3.4 Antibacterial activity

The antibacterial activity was tested against pathogenic bacteria such as *E. coli spp.* and *Enterobacter cloaceae*, which are non-desirable in a large presence in the gut microflora [36, 37] as well as the positively associated bifidobacteria [38]. A concentration range 100 – 12.5 μg/mL was considered, which is similar or lower than described in comparable studies [7, 8]. For all strains used a similar effect of the non-roasted and roasted cocoa fractions could be observed (Figs. 6 and 7). Up to the concentration of 50 μg/mL there was no significant influence on the optical density, which means that there was no real growth reduction. In the *E. cloaceae*, however, there was a slight increase in the bacteria growth at these concentrations. For the highest 100 μg/mL solution, independent of the fraction and the roasting stage, bacteria growth was significantly decreased in each strain ($p < 0.05$). The highest reduction of the *E. cloaceae* was shown for the lowest MW fraction of < 5 kDa, for both the roasted and non-roasted fraction (Fig. 7). The lowest reduction was observed for the *E. coli* O157:H7 ATCC 43888, where the difference in optical density between the highest and lowest concentration was not significant. Unfortunately, the same growth-reducing effect was shown for the positive bifidobacteria strains Bb-12 and B 7.1. For both strains, the highest concentration reduced the growth rate significantly, the Bb-12 was susceptible to the 25 and 50 μg/mLconcentrations, too.

In the agar diffusion-well assay, no fraction of the tested cocoa samples formed clear zones.

Antibacterial activity of MRP was also shown for the lysozyme-glucose stearic acid monoester conjugate formed through the Maillard reaction [39] at the optimal concentration of 50 μg/mL. Ames *et al*. [9] showed that melanoidins isolated from a carbohydrate-amino acid model could be used as a carbon source for human large-bowel bacteria. Very recently, Hiramoto *et al*. [40] found a suppression of *Helicobacter pylori in vitro* as well as *in vivo* by melanoidins formed from casein and lactose. Food-based melanoidins were only tested so far on prebiotic activity for bread crust [7] and coffee silverskin [8]. For both latter studies, similar results have been observed. However, as they tested one concentration at different fermentation times, a real comparison is not possible.

The results, presented here, prove the antibacterial activity of cocoa melanoidins of different MW fractions. However, not only pathogenic but also beneficial bacteria were suppressed in their growth activity.

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