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# Bread crust melanoidins as potential prebiotic ingredients

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Melanoidins are the final products of the Maillard reaction. They are a heterogeneous mixture of compounds characterized by brown color and high molecular weight. The physiological properties of melanoidins have been widely investigated and there is a general consensus on their poor digestibility and bioavailability. *In vitro* studies on food melanoidins are in many cases limited by their poor water solubility. This problem was recently overcome for bread melanoidins using an enzymatic digestion procedure. Bread melanoidins are constituted by low-molecular-weight, colored compounds linked to the gluten polymer. In this work, melanoidins from different bread types were investigated for their potential prebiotic activity by a static batch culture. Results showed that anaerobic bacteria, particularly Bifidobacteria strains, are able to use bread melanoidins as carbon source. The bacterial growth is different for the various types of melanoidins samples indicating that starting materials and processing conditions have a strong influence on the prebiotic potential of bread melanoidins. In all cases the bacterial growth obtained using bread melanoidins is lower than that previously observed using melanoidins from other sources, such as coffee silverskin.

**Keywords:** Bifidobacteria / Bread melanoidins / Prebiotic capacity

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## 1 Introduction

The Maillard reaction (MR) normally occurs during thermal treatment of food containing reducing sugars and free amino groups. The extent of the reaction depends on the processing conditions, mainly on time, temperature, and water activity [1]. Using relatively severe conditions the formation of high-molecular-weight brown compounds called melanoidins is obtained. Little information is known on these polymers particularly regarding their structure and biological properties. The chemical structure of melanoidins is not completely elucidated, but, in the last few years, more data became available. In particular, it was shown that they can have a different structure according to the different starting material: in some cases they are mainly formed by a carbohydrate skeleton with few unsaturated rings and a small nitrogen component, in other cases they can have a protein structure linked to small chromophores. Several studies pointed out the fundamental technological role played by melanoidins in foods (texture, binding of volatiles, antioxidant action); on the other hand, our knowledge on their metabolic fate after dietary ingestion is still in its infancy. Different studies on animals showed that melanoidins could escape digestion and pass through the upper gastrointestinal tract [2, 3]. Subsequently, they may be susceptible to metabolism by the vast numbers of microorganisms present in the hindgut [4]. The equilibrium among the different species present in the gut microflora is responsible for the proper functioning of the body and many human pathologies have been correlated with intestinal dysmicrobism [5].

The prokaryotic cells normally present in the human gut include the following bacterial genera: Bifidobacteria, Clostridia, Bacteroides, Lactobacilli, Streptococci, and Enterobacteriacea. Many studies have shown that Bifidobacteria and Lactobacilli are desirable bacteria in the gut due to their health-promoting properties, such as inhibition of exogenous pathogens [6] and prevention of colon cancer [7]. Therefore, there is a general interest in finding dietary components able to promote the competitive growth of beneficial species *versus* the pathogenic one. These kinds of food ingredients are known as prebiotics (*i.e.*, promoting the growth of probiotic bacteria). Up to now many dietary fibers have been tested for their prebiotic activity, however, all dietary components escaping digestion and absorption in the first part of the gastrointestinal tract can be metabolized by a lower gut microflora. Emerging evidence indicates that also other components, beside dietary fiber, can

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**Abbreviation:** **MR** Maillard reaction

increase the growth of beneficial bacteria, in particular the dietary fiber-associated polyphenols [8]. Data from human and animal studies suggested that dietary melanoidins are not digested in the upper gastrointestinal tract and are mainly recovered in the feces [9]. Although melanoidins cannot be strictly considered dietary fiber, as they are formed upon food processing, melanoidins behave like dietary fiber as they are not digested in the upper gastrointestinal tract. The question if they are fermented by the intestinal microflora or not, is still open. Ames and coworkers [10] showed that melanoidins from a carbohydrate-amino acid model system can be used as carbon source and we have recently shown that coffee silverskin, a coffee by-product rich in melanoidins, displays the typical features of a potential prebiotic ingredient [11].

In this work, using a static batch culture system, a fermentation study using melanoidins from different bread crust samples has been performed. The aim was to investigate the fermentation properties of bread crust and to verify the hypothesis that compounds formed during thermal treatment can influence the growth of human intestinal microflora. To perform these experiments, a prerequisite is to obtain a sufficient amount of material in a water-soluble form. Water and organic solvents are not able to extract brown components to a significant extent; therefore, we have developed an enzymatic extraction procedure using bacterial proteolytic enzymes, which allowed us to obtain brown water-soluble material from bread crust [12].

## 2 Materials and methods

### 2.1 Materials

#### 2.1.1 Bread samples and chemicals

For our purposes three different Italian bread crust samples were purchased at a typical bakery. The bread samples were the following. Sample A (Schiacciatina): a 250 g size bread obtained from wheat meal and dough from a previous preparation (1 : 1 w/w). Proximate composition: 8.1% proteins, 65.2% carbohydrates, 0.5% fats, 2.8% dietary fibre. Sample B (San Sebastiano): a 1000 g size bread obtained from wheat meal and dough from a previous preparation (5 : 1 w/w). Proximate composition: 8.1% proteins, 66.1% carbohydrates, 0.3% fats, 3.1% dietary fibre. Sample C (Integrale): a 250 g size bread obtained from whole-meal, malt, and yeast. Proximate composition: 7.5% proteins, 55.8% carbohydrates, 1.3% fats, 6.2% dietary fibre. All breads were baken at the same temperature (220°C), samples A and C for 20 min, sample B for 1 h. Pronase E from Streptomyces griseus was from Fluka (Milan, Italy) while buffers and other products were obtained from Sigma-Aldrich (Poole, Dorset, UK). Bacteriological growth media were from Oxoid (Basingstoke, UK).

#### 2.1.2 Apparatus

A laboratory blender (Waring Commercial) was used to reduce the bread crust to a fine powder. The centrifuge, utilized to separate digested crust from the indigestible product, was a Jouan CR3i (BICASA S.p.A.) while an Amicon ultra-filtration kit (Millipore, Bedford, MA, USA) provided a YM-10 (RC) membrane. Bacteriological growth media were autoclaved before use. The fermenters were incubated in an anaerobic chamber (Don Whitley Scientific, Shipley, West Yorkshire, UK) under an atmosphere of $N_2$-$CO_2$-$H_2$ (80 : 10 : 10 v/v/v) at 37°C and in an aerobic chamber at 37°C.

### 2.2 Methods

#### 2.2.1 Preparative phase

##### 2.2.1.1 Sample preparation

All samples were prepared in triplicate as described by Borrelli *et al.* [12]. Sample preparation was performed by adding 6 mL 0.2 M Tris-HCl, pH 8.0, containing Pronase E (0.1 mg/mL) to 500 mg freeze-dried bread crust; the solutions were kept at 37°C for 70 h. The brown supernatants, containing the hydrolysed products, were recovered after centrifugation at 4000 rpm for 15 min. Separation of compounds with high molecular weight (cut-off > 10 000) from those with low molecular weight (cut-off < 10 000) was obtained by ultrafiltration. Finally all high-molecular-weight fractions containing the melanoidins were freeze-dried and stored at −20°C until use.

##### 2.2.1.2 Bacteriological growth media

All agar substrates were prepared in triplicate following the procedure of Olano-Martin *et al.* [13]. The substrates were: Nutrient agar (NA) for total aerobes, Wilkin's Chalgren (WC) for total anaerobes, Mac Conkey agar No. 3 (MC) for Enterobacteriacea, Rogosa agar (RG) for Lactobacilli, reinforced clostridial agar (CL) for Clostridia, Beeren's agar (BR) for Bifidobacteria, and Brucella agar (BC) for Bacteroides. Colonies developed at the end of the experiments were counted manually.

##### 2.2.1.3 Microbiological culture techniques

Freshly voided feces were always used to prepare the inoculum for the batch cultures. One mL of batch culture was serially diluted from $10^{-1}$ to $10^{-9}$. After thorough mixing, 20 µL was plated onto one quarter of an agar plate, four dilutions per plate, onto triplicate plates. Ten selective media were used as reported in Table 1. Plates were then

 

**Table 1.** Selected growth media and bacterial groups enumerated

| Selective growth media | Dilutions plated | Target group |
|---|---|---|
| Nutrient agar | $10^{-1}-10^{-5}$ | Total aerobes |
| MacConkey agar No. 3 | $10^{-1}-10^{-4}$ | Coliforms |
| Wilkens-Chalgren agar | $10^{-4}-10^{-7}$ | Total anaerobes |
| Bacteroides agar | $10^{-4}-10^{-7}$ | Bacteroides spp. |
| Beerens agar (BA) | $10^{-4}-10^{-7}$ | Bifidobacterium spp. |
| Azide/Crystal violet agar | $10^{-1}-10^{-4}$ | Enterococcus spp. |
| Rogosa agar | $10^{-0}-10^{-7}$ | Lactobacillus spp. |
| Fusobacterium agar | $10^{-2}-10^{-5}$ | Fusobacterium spp. |
| Raffinose-Bifidob. agar (BR) | $10^{-4}-10^{-7}$ | Bifidobacterium spp. |
| Clostridia agar | $10^{-3}-10^{-6}$ | Clostridium spp. |

aerobically incubated at 37°C for 24 h – 72 h (Nutrient, MacConkey, and azide/Crystal violet agars) or anaerobically 72 h – 120 h (Wilkens-Chalgren, Bacteroides, Beeren's, Raffinose-Bifidobacterium, Fusobacterium, Rogosa, and Clostridial agars). Bacterial colonies were differentially counted, according to morphology, using a colony counter, summed, and the total log counts per mL of sample calculated. Colonies were tentatively identified using morphotype and selected microscopic/biochemical traits as described by Gibson and Roberfroid [5]. All procedures were carried out in an anaerobic cabinet (10% $H_2$; 10% $CO_2$; 80% $N_2$).

#### 2.2.1.4 Chemostat basal media

Nutrient media were prepared as described by Olano-Martin *et al.* [13]. All substrates and nutrient media were autoclaved and put in the anaerobic cabinet overnight while still hot.

#### 2.2.1.5 Inoculum

Three different healthy donators, who had not taken antibiotics for 3 months before, provided fresh fecal material. A 10% w/v dilution of the fecal sample in sterile, pre-reduced PBS was used as inoculum for the batch culture. The final concentration of the fecal slurry in the batch cultures was 1% w/v.

### 2.2.2 Experimental phase

#### 2.2.2.1 Fermentation of melanoidins in batch cultures of mixed fecal bacteria

The ability of bacteria to utilize bread melanoidins (A-B-C) and glucose (control-E) were performed as described by Olano-Martin *et al.* [13] using the static batch culture fermenters (in an anaerobic cabinet at 37°C). 0.5 g of each melanoidin (or 0.5 g glucose as control) were dissolved in 5 mL PBS at pH 7.0 and then added 44 mL chemostat basal media and 1 mL fecal slurries were added. A 5% w/v dilution of the fecal sample in sterile PBS was used as inoculum for the batch culture. The final concentration of the fecal slurry in the batch cultures was 1% w/v. Each sample (1 mL) was removed after 0, 5, 10, and 24 h for bacteriological analysis, in triplicate, on the selective plating media previously prepared to isolate specific microorganisms. The whole experiment for each sample was performed in triplicate using every time a different donator of fecal inoculum. Subsequently the microbial growth on bread melanoidins was measured by colony count on selective agars. The identification of microorganisms was performed using Gram stain and microscopy.

#### 2.2.2.2 Statistic analysis

The data obtained (number of colonies and dilution value) were processed applying the formula: Log (count × 50) × $10^{dil}$, where count is the number of colonies, 50 is the volume (mL) of nutrient media + sample, and dil is the dilution of count. Differences between bacterial counts at 24 h fermentation for each substrate were tested for significance using the Tukey test, which allowed a multiple comparison among the data to individualize the significant difference among the same. Differences were considered significant if $P \leq 0.05$.

## 3 Results

The crust of three different types of breads was used to extract, by enzymatic digestion, the melanoidins formed during cooking. The enzymatic digestion procedure, allowed us to obtain a sufficient amount of water-soluble bread crust melanoidins to run in batch culture fermentations. It is worth noting that the extraction of bread crust with water or organic solvent resulted in a very limited melanoidin solubilization. The enzymatic hydrolysis proves to be an essential step to generate suitable material for the fermentation work. It results in the formation of a dark-brown solution (absorbances at 360 nm of 0.15, 0.22, and 0.34 for sample A, B, and C, respectively). The color of the solution is mostly retained after ultrafiltration in the high-molecular-weight components, as previously observed [12]. The yield of each water-soluble melanoidin obtained by enzymatic digestion from bread crust was about 30%. The samples were then used for the static batch cultures in experiments aimed to evaluate if the intestinal microflora is able to use bread melanoidins as carbon source.

During this study, the procedures described in Section 2 with fecal inocula from three different donors were used and the result of the fermentation on bread crust melanoidins was measured. Samples were taken from the batch cultures after 0, 5, 10, and 24 h of fermentation. The time course of the growth of six groups of bacteria was consid-

 

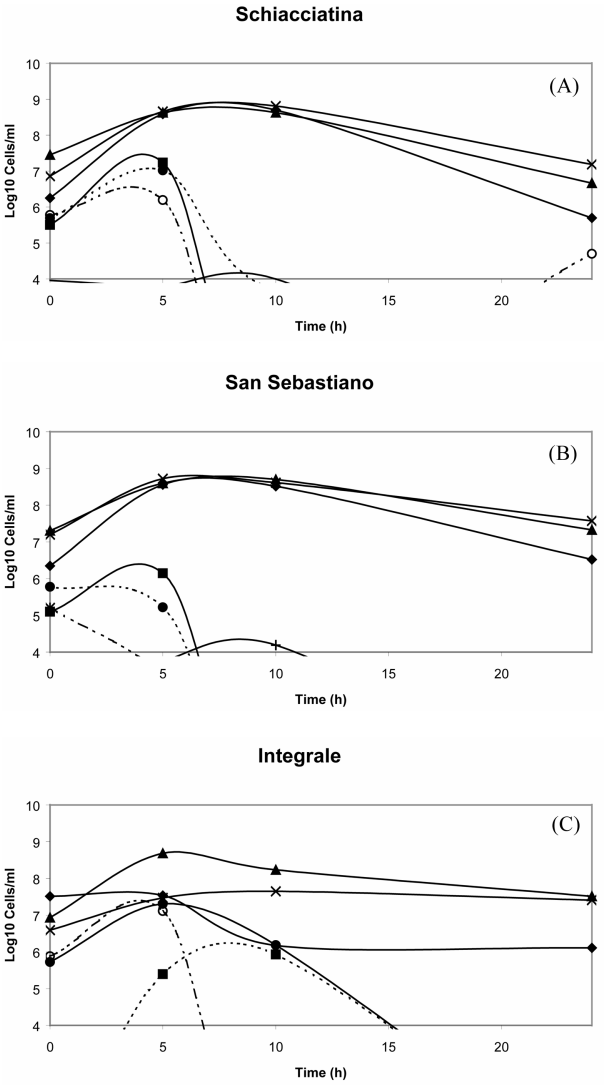


**Figure 1.** Bacterial growth after 24 h of fermentation using the three different bread melanoidins (panel A, B, and C, respectively). (▲) Total anaerobes; (♦) total aerobes; (×) Bifidobacteria; (○) Bacteroides; (●) Clostridia; (■) coliforms. The number of Lactobacilli cells is <4 (Log cell/mL), so their growth is not visible on the graph.

ered to evaluate the ability of gut microflora to use this material as carbon source. Selective substrates were used to monitor the growth of the following strains: Bifidobacteria, Lactobacilli, Clostridia, Bacteroides spp., Streptococci and the Enterobacteriacea. The first two bacterial groups have been associated with a healthy intestinal condition, while Clostridia and Bacteroides spp. are considered as potentially dangerous members of the gut microflora. Also the total aerobes and total anaerobes were monitored.

As shown in Fig. 1, an overall increase in the number of microorganisms was observed during fermentation with the three types of bread melanoidins. In particular, the increase of the numbers of total anaerobic and aerobic bacteria demonstrates the ability of microorganisms to use the bread melanoidins as sources of carbon and nitrogen. Comparing the bacterial counts on selective growth media it is possible to observe a predominant development of Bifidobacteria with respect to the other microorganisms. In fact, Enterobacteriacea, Bacteroides spp., and Clostridia showed a limited growth after 10 h. On the other hand, Lactobacillus spp. showed a poor aptitude to use bread melanoidins for their growth. Beside the ability to growth on the selective media, the presence of Bifidobacteria was confirmed by Gram staining and by analysis with an electronic microscope confirming the presence of pleomorphic rods typical for Bifidobacteria strains.

Figure 2 shows a comparison of the growth, after 24 h of fermentation, of the different bacterial species on the three types of bread melanoidins compared with the same concentration of glucose used as control. The graphic shows the predominance of Bifidobacteria among the anaerobic species. Comparing the results, it is possible to observe that the number of total anaerobic bacteria is quite similar in all tested samples, but the C sample gave the highest growth of Bifidobacteria also in comparison with the control. Moreover, sample B was able to support the growth of a high number of aerobic bacteria but not of Enterobacteriacea. After Gram staining, these colony morphotypes were observed at the electronic microscope and identified as Streptococcus spp. (rods and diplococci), which are common facultative anaerobes in the human gut microflora.

## 4 Discussion

The *in vitro* experimental conditions used in this study were selected to mimic the hindgut environment. The experiments were performed in an anaerobic cabinet to create the same atmosphere present in the intestine, and serum bottles containing nutrient media and fecal bacteria represented the lumen. The data demonstrate that bread crust melanoidins can be used as carbon and nitrogen source by the hindgut microflora. In particular, it is possible to note that after about 10 h of fermentation bread melanoidins well support Bifidobacteria growth, while the other bacteria considered (Clostridia, Bacteroides spp., Streptococci, and Enterobacteriacea) showed a limited aptitude to use melanoidins as carbon source. Although the experimental plan used in this study, namely the colony morphology and microscopial cell shape, are not sufficient to address unknown isolates as Bifidobacteria, our study represents the first screening to evaluate the potential prebiotic activity of bread crust melanoidins.

In a parallel study we have applied the static batch culture approach also to another type of melanoidins, namely the

 

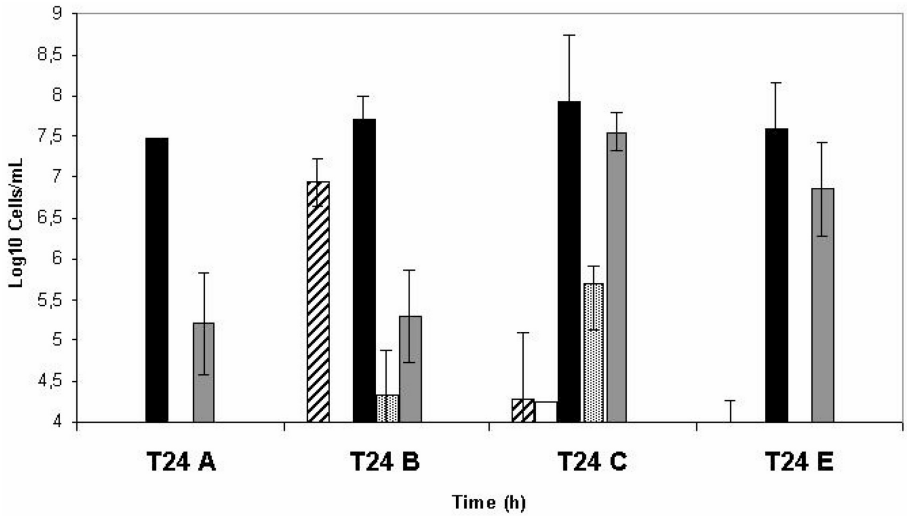


**Figure 2.** Bacterial growth after 24 h (T24) of the three different bread melanoidins (T24A, T24B, T24C) and glucose used as control (T24E). Black bars, total anaerobes; gray bars, Bifidobacteria; white bars, bacteroides; shaded bars, total aerobes; dotted bars, coliforms. Clostridia and Lactobacilli growth are < 4 (Log cell/mL).

high-molecular-weight brown fraction from coffee silverskin, a by-product of coffee bean roasting [11]. The growth of Bifidobacteria using coffee silverskin was more pronounced than that observed using bread melanoidins. The result is likely due to the completely different nature of the two types of melanoidins, namely bread melanoidins characterized by a considerable protein structure and coffee melanoidins with a prevalence of the carbohydrate moiety end with the presence of phenolic residues.

Different studies have shown that Bifidobacteria induce positive consequences for host health and that certain food ingredients are able to preferentially support the growth of Bifidobacteria and Lactobacilli *in vitro* and *in vivo* [5]. Melanoidins are formed by a substantial portion of carbohydrates, whose digestion is limited by the cross-linked bonds generated by thermal treatment through MR. Therefore, it is conceivable that in the lower gut food melanoidins have physiological functions similar to those of dietary fibers.

Summarizing, the experiments led to two main indications: (i) bread crust melanoidins can be metabolized/fermented by the human hindgut microflora; (ii) bread crust melanoidins seem to selectively enhance the growth of bifidobacteria, therefore, they may have the potential prebiotic activity similar to that of dietary fibers. However, it is not possible to reach a general conclusion on prebiotic effects of food melanoidins. In fact, considering the wide range of possibilities given by raw material and processing conditions, the specific effect of each different food melanoidins should be investigated.

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