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Phytic acid degrading lactic acid bacteria in tef-injera fermentation

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ABSTRACT

Ethiopian injera, a soft pancake, baked from fermented batter, is preferentially prepared from tef (*Eragrostis tef*) flour. The phytic acid (PA) content of tef is high and is only partly degraded during the fermentation step. PA chelates with iron and zinc in the human digestive tract and strongly inhibits their absorption. With the aim to formulate a starter culture that would substantially degrade PA during injera preparation, we assessed the potential of microorganisms isolated from Ethiopian household-tef fermentations to degrade PA. Lactic acid bacteria (LAB) were found to be among the dominating microorganisms. Seventy-six isolates from thirteen different tef fermentations were analyzed for phytase activity and thirteen different isolates of seven different species were detected to be positive in a phytase screening assay. In 20-mL model tef fermentations, out of these thirteen isolates, the use of *Lactobacillus* (*L.*) *buchneri* strain MF58 and *Pediococcus pentosaceus* strain MF35 resulted in lowest PA contents in the fermented tef of 41% and 42%, respectively of its initial content. In comparison 59% of PA remained when spontaneously fermented. Full scale tef fermentation (0.6 L) and injera production using *L. buchneri* MF58 as culture additive decreased PA in cooked injera from 1.05 to 0.34 ± 0.02 g/100 g, representing a degradation of 68% compared to 42% in injera from non-inoculated traditional fermentation. The visual appearance of the pancakes was similar. The final molar ratios of PA to iron of 4 and to zinc of 12 achieved with *L. buchneri* MF58 were decreased by ca. 50% compared to the traditional fermentation. In conclusion, selected LAB strains in tef fermentations can degrade PA, with *L. buchneri* MF58 displaying the highest PA degrading potential. The 68% PA degradation achieved by the application of *L. buchneri* MF58 would be expected to improve human zinc absorption from tef-injera, but further PA degradation is probably necessary if iron absorption has to be increased.

1. Introduction

Injera, the staple food consumed widely in Ethiopia (Umeta et al., 2005), is a pancake prepared from tef (*Eragrostis tef*), an ancient cereal, indigenous to Ethiopia (Zegeye, 1997). Traditional preparation of Ethiopian tef-injera has not changed over decades (Stewart and Getachew, 1962; Yetneberk et al., 2004) and involves a fermentation step of 2–3 days based on continuous backslopping, a process in which the new batter is inoculated with a leftover from the previous fermentation, called "ersho" in Amharic. Ersho, tef flour and water are thoroughly kneaded and allowed to rest. Liquid that gets separated on top is discarded and replaced with fresh water. Before the batter is finally poured onto a clay plate and baked, a so-called "absit" is prepared by boiling a portion of the batter with water and adding it back to the fermenting batter, ensuring the desired textural quality of the baked injera pancake. Due to the use of ersho, the pH drops rapidly (Yigzaw et al., 2004) and reaches values below pH 4 within hours (Baye et al., 2013; Stewart and Getachew, 1962). As would be expected with acidification, microbial analysis of tef-injera batter has reported lactic acid bacteria (LAB) to be the major fermentative microbes accompanied by yeast, *Enterobacteriaceae* and *Bacillus* spp., but the latter was detected to a lesser extent (Nigatu and Gashe, 1998). These findings are comparable to other sourdough preparations (Minervini et al., 2014).

Tef is rich in phytic acid (PA), *myo*-inositol hexakisphosphate, which is the main phosphate storage form in most cereals, legumes and nuts. With its 6 phosphate groups, PA has a large number of negatively charged residues over a broad pH range and a strong chelating potential for divalent cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+} in the human digestive tract (Schlemmer et al., 2009). The PA-mineral chelates are insoluble and prevent the absorption of nutritionally important minerals and trace elements from plant-based foods (Hurrell, 2004). This is a major concern in developing countries, where cereals and legumes are the major source of minerals for large population groups, and where plant based diets are associated with nutritional iron and zinc deficiencies (Ramakrishnan, 2002).

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Several strategies are available to overcome the inhibition of PA on iron absorption, including the addition of ascorbic acid or EDTA to meals (Hurrell, 2002), but these strategies do not improve zinc absorption. One promising measure that has been reported to increase both iron and zinc absorption from foods is enzymatic degradation of PA (Egli et al., 2004; Troesch et al., 2009). Phytases hydrolyze inositol phosphates and have been reported in microorganisms, plants and animals, although their activity and optimum conditions for reaction differ widely (Konietzny and Greiner, 2002). The selection of an appropriate enzyme adapted to the processing conditions of a specific food is therefore critical. Phytases are present in cereals (Egli et al., 2002) but have not been reported in tef, although PA has been reported to be partially degraded during tef-injera fermentation (Abebe et al., 2007; Umeta et al., 2005; Urga and Narasimha, 1998). Although PA degradation has been reported to occur to varying extents, the loss is only partial unlike the almost complete reduction reported when wheat is added to injera preparation (Baye et al., 2013).

In addition to cereal phytases, microbial phytases can degrade PA during fermentation. Effective PA degradation was achieved by yeasts, isolated from Tanzanian togwa, during togwa fermentation (Hellström et al., 2012). But for the application of microbial phytase producers as starter culture in food production the safety aspect should not be neglected. This is why LAB, which are involved in many food processes and also have been noted in injera fermentations (Gashe, 1985) are of high interest as starter cultures. Selected strains of LAB species originating from European sourdough fermentations for bread production as well as from an African pearl-millet fermentation for gruel making have been reported to be capable of degrading PA (De Angelis et al., 2003; Lopez et al., 2000; Songré-Ouattara et al., 2008).

The present study was designed to identify and isolate microorganisms from tef-injera fermentation that have phytase activity and are eligible as starter culture for tef-injera fermentation. LAB, dominating in Ethiopian tef-injera fermentations, were isolated and screened for phytase activity. The LAB isolates showing PA degrading potential were characterized and further tested in model tef fermentations as well as in full scale tef-injera preparation for their ability to degrade PA within the food-matrix.

2. Materials and methods

2.1. Chemicals and flours

Unless otherwise specified, chemicals of p.a. grade were purchased from Sigma-Aldrich Chemie GmbH, Switzerland and Merck KGaA, Germany. Media and components for cultivation of microbes were ordered from Becton Dickinson AG, Switzerland, if not stated differently. Tef for fermentations performed at the laboratory in Zurich was bought at a local market in Debre Zeyit (Bishoftu), Ethiopia and milled to wholegrain flour in a mill at the same location.

2.2. Phytase application

Aspergillus niger phytase euphoVida™ 20000G was kindly provided by DSM Nutritional Products, Kaiseraugst, Switzerland. To ensure complete PA degradation during 48 h of tef fermentation (see Section 2.7), double the amount of phytase units (380 FTU/g PA) theoretically needed to degrade all the PA was applied to tef flour. The enzyme is inactivated by baking.

2.3. Sampling of Ethiopian tef-injera batter

The traditional injera preparation at household level was studied in the Debre Zeyit (Bishoftu) area, which is situated on the elevated plain of central Ethiopia. Families from the urban and surrounding rural areas who were willing to demonstrate their way of tef-injera preparation were selected. The tef flour used by the families was from their own stocks, either bought at a local market or grown on their own land. For later enumeration and isolation of microorganisms, approximately 5 g fermenting batter (before the absit preparation step) was collected in duplicate from each family in a sterile falcon tube prefilled with 1 mL glycerol. The samples were stored on ice during the study-day, before the final storage at −20 °C.

2.4. Enumeration of microbes

At ETH Zurich, thawed Ethiopian tef-injera batter samples from 6 households were analyzed in duplicate for total microbial content and a further 7 tef-injera batter samples for LAB only. To evaluate the model fermentations performed at the laboratory in Zurich, samples of about 1 g fresh batter were taken for immediate analysis.

All batter samples were diluted tenfold (w/w) in peptone-solution and 0.1 mL of subsequent serial ten-fold dilutions were spread on selective agar media in duplicates for enumeration of bacteria and fungi. Presumptive lactobacilli were selected on MRS agar medium (1.5% agar), incubated anaerobically (AnaeroGen packs, Oxoid, UK) at 37 °C. YM agar medium with chloramphenicol (100 mg/L) against bacterial growth was used to cultivate yeasts at 25 °C. General bacterial counts were performed on aerobic plate count (PC) agar medium at 30 °C as well as presumptive *Bacillus* spp. spore counts after a heat treatment step at 85 °C for 15 min (Kastner, 2008). Presumptive enterococci were enumerated on KFS agar medium incubated at 43 °C for 48 h. VRBD agar medium was used to detect *Enterobacteriaceae* after a 48 h incubation at 37 °C. After incubation, cell counts were calculated in colony forming units (cfu) per g of batter.

2.5. Isolation, cultivation and characterization of LAB

Dominant, presumptive LAB were picked (2–3 isolates per colony morphology) from MRS agar media, containing between 30 and 300 cfu obtained from diluted injera batter, and were purified by repeated streaking on the same medium. Strains were assessed for Gram-classification (3% KOH), catalase activity (3% H_2O_2), and by phase contrast light microscopy. Liquid cultures were grown in MRS media and stored at −80 °C in 25% (v/v) glycerol as a cryoprotectant.

For DNA extraction from LAB, a single colony was picked and suspended in 0.1 mL buffer and treated further as Goldenberger et al. described in procedure B (Goldenberger et al., 1995). For later amplification, the final DNA preparations were kept at −20 °C.

To differentiate the particular LAB strains used, rep-PCR fingerprints were produced by DNA amplification with the $(GTG)_5$ primer (Gevers et al., 2001). PCR products were visualized by UV light on ethidium bromide stained agarose (1.8%) after gel electrophoresis in 1× TAE-buffer (pH 8.0), using 1-kb and 100-bp DNA ladders (Generuler, Fermentas GmbH, Switzerland) as reference.

To follow the fate of applied LAB starter strains in fermentations, 2–4 colonies from the two highest injera batter dilutions were randomly picked from MRS agar medium and analyzed by rep-PCR fingerprints as described above.

The isolates were identified to the species level by partial 16S rRNA gene sequencing. These regions were amplified with the help of the primer pairs bak4 (5′-AGG AGG TGA TCC ARC CGC A-3′) (Greisen et al., 1994)/bak11w (5′-AGT TTG ATC MTG GCT CAG-3′) (Goldenberger et al., 1997) or 7f (5′-AGA GTT TGA TYM TGG CTC AG-3′)/1510r (5′-ACG GYT ACC TTG TTA CGA CTT-3′) (Nielsen et al., 2007) based on a protocol described by Dasen et al. (1998) and Nielsen et al. (2007), respectively. The product was purified (GFX PCR DNA & Gel Band Purification Kit, GE Healthcare, UK) and prepared for standard Sanger sequencing reactions performed at GATC-Biotech (Konstanz, Germany). The resulting sequences were edited in FinchTV (version 1.4, Geospizza Inc., USA), assembled with the help of BioEdit (version 7.0.9, Ibis Biosciences, USA) and compared to sequences reported in GenBank (NCBI, USA), using the blastn algorithm.