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CELINA EUGENIO BAHULE

METAPROTEÔMICA COMO FERRAMENTA TECNOLÓGICA PARA OTIMIZAÇÃO DOS PARÂMETROS DE FERMENTAÇÃO DO MILHO PARA FORMULAÇÃO DE PASTA ALIMENTíCIA

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Orientadora: Profa. Dra Alessandra Santos Lopes

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DEDICATÓRIA

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Resumo

Na maioria dos países em desenvolvimento produtos de milho são muitas vezes os que aliviam a fome e garantem a subsistência da população. A fermentação é um meio de processamento usado para amolecer o grão de milho, melhorar o sabor e a digestibilidade, além de que alguns produtos de milho fermentado são utilizados com a crença de terem propriedades funcionais. A pasta de milho fermentada foi relatado como tendo efeitos probiótico e nutrancêutico, contudo, outros estudos desencorajam o consumo desta, justificando pela carga e diversidade de microrganismos surgidos durante a fermentação. Com o objectivo de estudar o perfil funcional e procurar prováveis caminhos para otimização da pasta de milho, foi feito uma recriação da fermentação espontânea do milho, e, formulada uma pasta alimentícia que foi submetida à análise metaproteômica. Um total de 53977 peptídeos foram recuperados e identificados, em que mais de 60% foram associados ao substrato (milho), 6% a fungos e 34% às bactérias. A metaproteômica revelou uma provável associação das Actinobacteria com as enzimas de degradação do amido da matriz. Proteobacterias (maioria patogenicos) e Firmicutes (parte probióticas) coexistiram e se multiplicaram em igual proporção até o final, aliado a isso, a contagem alta de mesófilos (213 x 10^6 UFC/g) e a redução timida do pH (4.42) sugeriram um produto alimentício não totalmente seguro. Como forma de otimizar o processo, o milho foi autoclavado a 121ºC por 20 min para inativação enzimatica e eliminação de microrganismos, e então inoculado com *Pediococcus acidilati.* Milho não tratado foi também inoculado. O desempenho da cepa tanto no milho pré-tratado (T+I) quanto no milho não tratado (nT+I) foi considerada ótima, pois logo nas primeiras 24 horas os valores de pH, 4.2 para as amostras nT+I e 4.41 para amostras T+I estavam em níveis considerados seguros, e reduziram com o tempo de fermentação. A correlação forte entre os principais parametros de fermentação quando o *P.acidilati* foi utilizado para inoculação do milho abre possibilidades para aplicação de modelos de otimização rumo a produção rápida, segura e industrial da pasta de milho. A metaproteômica como técnica livre de cultivo e baseada em proteínas, permite a análise e identificação rápida da microbiota , com a vantagem de exibir e correlacionar o perfil microbiano com a funcionalidade.

Palavras chave: pasta de milho; fermentação lactica; proteômica; perfil microbiano; otimização.

Abstract

In most developing countries, maize products are often what alleviate hunger and guarantee the livelihood of the population. Fermentation is a processing medium used to soften the corn kernel, improve flavor and digestibility, and some fermented corn products are used in the belief that they have functional properties. Maize dough, obtained by fermentation, has already been reported as having a probiotic and nutraceutical effect, however, other studies discourage its consumption, justifying it by the load and diversity of microorganisms that develop during fermentation. With the aim of studying the functional profile and looking for likely ways to optimize maize dough, a recreation of the spontaneous fermentation of corn was performed, and the formulated dough was subjected to metaproteomics analysis. A total of 53977 peptides were recovered and identified, of which more than 60% were associated with the substrate, 6% with fungi, and 34% with bacteria. Metaproteomics revealed a probable association between Actinobacteria and starch degradation enzymes in the matrix. Proteobacteria (pathogenic majority) and Firmicutes (probiotic part) coexisted and multiplied in equal proportion until the end, allied to this, the high count of mesophiles (213 x 10^6 CFU/g) and the timid reduction of the pH (4.42) suggested a food product not completely safe. As a way to optimize the process, the maize was autoclaved at 121 ^oC for 20 min for enzymatic inactivation and elimination of microorganisms, and then it was inoculated with *Pediococcus acidilati.* Untreated maize was also inoculated. The performance of the strain both in pre-treated maize (T+I) and in untreated maize (nT+I) was considered excellent, since, in the first 24 hours, the pH values, 4.2 for nT+I samples and 4.41 for T+I samples were at levels considered safe and decreased with fermentation time. The strong correlation between the fermentation parameters when *P. acidilati* was used for maize inoculation, opens possibilities for the application of optimization models towards the fast, safe, and industrial production of maize dough. Indeed, metaproteomics, as culture-free and protein-based technique, allows the rapid analysis of the microbiota and the identification of active microorganisms, with the advantage of displaying and correlating the microbial profile with functionality, and are well indicated to study fermented foods quickly and concisely, demonstrating effective methods for optimization.

Keywords: maize dough; lactic fermentation; proteomics; microbial profile; optimization.

LISTA DE SIMBOLOS, SIGLAS, ABREVIAÇÕES E UNIDADES

AGV´s	Ácidos graxos voláteis
ANOVA	Análise de variância
AOAC	Association of Official Analytical Chemists
LAB	lactic acid bacteria
DNA	Deoxyribonucleic acid
GRAS	Generally recognized as safe
ha	Hectares
HPLC	High-performance liquid chromatography
ITEC	Instituto de Tecnologia
ITV	Instituto de Tecnologico Vale
LABIOTEC	Laboratório de Processos Biotecnológicos
LTR4	Receptor toll - 4 like
pH	Potencial hidrogeniônico
RNA	Ribonucleic acid
Ton/ha	Toneladas por hectare
UFC	Unidade formadora de colônia
UFPA	Universidade Federal of Pará

Sumario

1 INTRODUÇÃO GERAL

Alimentos à base de milho são consumidos em diversas partes da Ásia, América central e em todas regiões da África. Na África o milho é alimento básico e na maioria dos países são os produtos de milho que aliviam a fome e garantem a nutrição e saúde. A forma de preparo varia de região para outra, podendo se destacar as massas espessas, mingaus, bebida energética e pastas (PHIRI, 2019). A pasta de milho é um alimento tradicionalmente formulado, cujo preparo é antecedido por uma fermentação. Grãos de milho inteiros, quebrados ou moídos são colocados para fermentar em água por um ou mais dias, com o principal objetivo de amolecer o grão e melhorar o sabor e a digestibilidade . Na maioria das comunidades africanas a pasta de milho fermentada é fornecida como primeiro alimento para bebês, ou como alimento para crianças pós desmame, seu consumo é também encorajado para mulhres grávidas e lactantes, assim como para doentes em convalescença. O fornecimento destes produtos de milho fermentado para categorias sensíveis da população é sustentada pela fácil digestibilidade e alta palatabilidade, e ao possível combate aos problemas entéricos (OGODO et al., 2017). Com efeito, alguns estudos relatam propiedades funcionais probiotico e nutrancêutico de produtos de milho fermentado (CHAVES-LÓPEZ et al., 2020b). Controversialmente, outros estudos relatam o efeito contrário e o perigo do consumo de produtos de fermentação do milho, devido a carga e diversidade de microrganismos neles contidos (CHIBUEZE IZAH et al., 2016; HANVI et al., 2021).

Diversos microorganismos colonizam o ambiente de fermentação do milho, atraídos pela rica composição de carboidratos do grão (amido, glicose, xilanas, hemicelulose e celulose), que servem de substrato apreciável (BAHULE et al., 2022). O facto é que nessa fermentação a dinâmica microbiana e sucessão de comunidades é incontrolável, evoluindo em função dos microrganismos presentes, do tempo de fermentação e da transformação do substrato (CHIBUEZE IZAH et al., 2016; GIRAFFA, 2004). Dada a importância de fermentar o milho para as comunidades, e da utilização dos produtos deste, mostra se necessário a realização de estudos para otimização do processo fermentativo e garantia da contínua utilização destes produtos com mais segurança e benefícios associados.

O uso de métodos convecionais para otimização do processo fermentativo demandaria um alto custo em tempo, recursos e conhecimento estatístico (GBASHI et al., 2020),

pois, vários estudos teriam que ser realizados, comecando pela inspeção, identificação e classificação dos microrganismos do produto fermentado, testagem de diferentes culturas iniciadoras e de diferentes modelos cinéticos da fermentação para encontrar o mais ajustado.

Atualmente, técnicas não convecionais de análise ajudam a encurtar o caminho, reduzir o custo e aumentar a precisão das pesquisas dos microrganismos em alimentos. São técnicas que dispensam o cultivo dos microrganismos, e, sua utilização pode ser associada as técnicas convencionais de forma complementar ou usadas de forma integral (FILIPPIS; PARENTE; ERCOLINI, 2017). Entre estas técnicas temos a eletroforese em gel de agarose (AGE), uma análise baseada em DNA microbiano e sequenciamento do gene 16S, ja foi aplicada em alimentos fermentados para estudar a sucessão da comunidade bacteriana (XIE et al., 2019b); e para identificar populações bacterianas importantes e selecionar as cepas mais adequadas para produzir dajiang (alimento oriental obtido por fermentação) de alta qualidade (XIE et al., 2019d).

Outras técnicas muito usadas, também livres de cultivo e baseadas em DNA e RNA são a metagenômica e metatrascriptoma respectivamente, permitem a análise da microbiota ativa, inativa, cultivável e não cultivável (FILIPPIS; PARENTE; ERCOLINI, 2017; WILSON; PIEL, 2013) Uma abordagem metagenômica foi usada para caracterizar os atributos taxonômicos e funcionais da microbiota de fermentação de huangjiu (LIU et al., 2020b), os autores mostraram que existe uma alta qualidade com esse procedimento e uma descrição científica em nível de gênero bem mais abrangente para refletir a caracterização da microbiota.

A técnica da metaproteômica baseadas em Proteinas, além da abordagem livre de cultivo, analisa os peptídeos e proteínas na amostra, com a vantagem de exibir o perfil de toda a microbiota cultivável, ativa e funcional, e qualquer atividade e funcionalidade dos peptídeos e proteínas (enzimas) relativos a estes. (RIZO et al., 2020), usaram a abordagem metaproteômica para analisar detalhadamente a fermentação do pozol (produto alimenticio de milho fermentado de origem mexicana), e inferir a partir das mudanças observadas nas proteínas, o papel funcional da microbiota durante a fermentação. Uma pesquisa feita sobre a aplicabilidade da abordagem metaproteômica para o processo de otimização da fermentação do milho, mostrou que esta tecnica era altamente recomendável e aplicável para estudo de alimentos fermentados (BAHULE et

al., 2022) Outras pesquisas desenvolvidas já mostravam o potencial da metaproteômica para analisar a funcionalidade de comunidades microbianas na rizosfera de milho e relataram esta como uma ferramenta poderosa, que usa dados de todas as proteínas nas amostras e fornece evidências diretas de diversidade e estrutura funcional e atividade microbiana entre a microbiota presente em nichos de qualquer ambiente (RENU et al., 2019)

O monitoramento do processo fermentativo é um fator de grande relevância para a indústria, uma vez que, pode determinar o tempo de fermentação necessário e a interferência de outros fatores que fazem parte do processo fermentativo. A determinação do tempo é relevante industrialmente, pois pode reduzir custos, tempo de processamento e gasto de energia nos reatores, sendo assim, o estudo da cinética e modelagem da fermentação vem como uma ferramenta adicional para o auxílio deste monitoramento (HASHEMI et al., 2021).

Modelos estatísticos podem ser aplicados para auxiliar a predizer a condição ideal dos parâmetros e a entender a influência do pré-tratamento sobre tais parâmetros. E também a obter o melhor desenho de acções futuras com vista a melhoria de todos aspectos da fermentação do milho que possam garantir a industrialização e com isso reduzir a desigualdade social e garantir a segurança alimentar e nutricional, principalmente entre os povos que mais consomem e dependem do milho.

A presente tese está organizada em capítulos, cada um representado por um artigo, sendo capítulo 1) artigo de revisão (já publicado); 2) artigo de resultados da aplicação da metaproteomica no estudo da dinâmica microbiana (já submetido) e 3) artigo sobre o estudo e aplicação de cepas iniciadora e pré-tratamento na fermentação do milho (em processo de submissão). A pesquisa foi realizada centrada nos seguintes objectivos:

1.1 Objetivo geral

Melhorar a produção e a segurança da pasta de milho utilizando a metaproteômica como ferramenta rapida e eficeinte para o estudo da otimização do processo fermentativo.

1.2 Objetivos específicos

Recriar a fermetação tradicional do milho e monitorar os parametros fermentativos e a carga da contagem microbiana dos microrganimos de importância alimentar.

Aplicar a metaproteomica sobre amostras da pasta de milho fermentada e observar o perfil e a funcionalidade das comunidades microbianas.

Selecionar e aplicar cepas iniciadores preditas como ideais pela metaproteomica e realizar a modelagem matemática ajustada à cinética dos parâmetros da fermentação para obter a melhor condição de fermentação.

General Introduction

Corn-based foods are consumed in many parts of Asia, Central America, and all parts of Africa. In Africa, maize is a staple food, and in most countries of Africa, it is maize products that alleviate hunger and ensure nutrition and health. The method of preparation varies from region to region, with thick dough, porridge, energy drinks, and soft dough (PHIRI, 2019) Maize dough is a traditionally formulated food that is prepared by fermentation. Whole, cracked, or ground corn kernels are soaked in water for one or more days, with the main aim of softening the kernel and improving flavor and digestibility through fermentation. Fermented maize dough is typically served as a baby's first food or as a child's first meal after weaning in most African cultures, as well as for the sick in convalescence, its consumption is also recommended for expectant and nursing mothers. The supply of these fermented corn products to sensitive categories of the population is supported to their excellent palatability, simple digestion, and potential to combat gastrointestinal issues (OGODO et al., 2017). Indeed, some studies report that fermented maize products have probiotic and nutraceutical benefits (CHAVES-LÓPEZ et al., 2020b). Controversially, some research suggest that consuming maize fermentation products has the opposite impact and poses a risk due to the quantity and variety of microorganisms they contain (CHIBUEZE IZAH et al., 2016; HANVI et al., 2021)

The rich carbohydrate composition of the maize grain (starch, glucose, xylans, hemicellulose, and cellulose), which acts as an important substrate, draws a variety of microbes to the maize fermentation environment (BAHULE et al., 2022). The microbial dynamics and succession of communities in this fermentation are uncontrollable and depend on the present microorganisms, the length of the fermentation, and how the substrate is changed (CHIBUEZE IZAH et al., 2016; GIRAFFA, 2004).

Given the importance of fermenting corn for this communities and the use of its products, it is necessary to carry out studies to optimize the fermentation process and guarantee the continuous use of these products with more safety and associated benefits.

The use of conventional methods to optimize the fermentation process would require a high cost in time, resources, and statistical knowledge (GBASHI et al., 2020), since several studies would have to be carried out, starting with the inspection, identification,

and classification of the microorganisms in the product fermented and testing different starter cultures and different kinetic models of fermentation to find the best fit.

Currently, non-conventional analysis techniques help to shorten the path, reduce the cost, and increase the accuracy of research on microorganisms in food. These techniques are free from microbial culture, and their use can be associated with conventional techniques in a complementary way or used in full (MORESI; PARENTE, 2014).

Among these techniques are agarose gel electrophoresis (AGE), an analysis based on microbial DNA, and 16S gene sequencing, which have already been applied to fermented foods to study bacterial community succession (XIE et al., 2019b) and to identify important bacterial populations and select the most suitable strains to produce high-quality dajiang (an oriental food obtained by fermentation) (XIE et al., 2019a).

Other widely used techniques, which are culture-free and based on DNA and RNA, are metagenomics and metatranscriptome, respectively, those one analyze active, inactive, cultivable, and non-culturable microbiota (WILSON; PIEL, 2013). A metagenomic approach was used to characterize the taxonomic and functional attributes of the Huangjiu fermentation microbiota (LIU et al., 2020a) . The authors showed that there is a high quality to this procedure and a much more comprehensive genus-level scientific description to reflect the characterization of the microbiota.

Another technique is a protein-based called metaproteomics, in addition to the culture-free approach, this technique analyzes the peptides and proteins in the sample, with the advantage of displaying the profile of all cultivable, active, and functional microbiota and any activity and functionality of the peptides and proteins (enzymes) relating to these. RIZO et al., (2021) employed the metaproteomics approach to thoroughly examine the fermentation of pozol, a fermented maize food product with Mexican origins, and deduce the functional role of the microbiota during fermentation from the observed changes in proteins. The use of the metaproteomics approach to the process of enhancing maize fermentation was examined in research, and it was discovered that this method was highly effective and suitable for the study of fermented foods (BAHULE et al., 2022).

The power of this tool, which uses information from all the proteins in the samples and provides direct evidence of diversity, functional structure, and microbial activity among

the microbiota present in niches in any environment, was also demonstrated in other developed research that examined the functionality of microbial communities in the corn rhizosphere (RENU et al., 2019).

Since it can estimate the required fermentation time and the influence of other fermentation components, the fermentation monitoring process is a very important factor for the industry. The study of fermentation kinetics and modeling provides an extra tool to enhance this monitoring, and time determination is industrially relevant as it can minimize costs, processing time, and energy expenditure in reactors (HASHEMI et al., 2021)

In order to improve all aspects of maize fermentation and ensure industrialization, which will reduce social inequality and ensure food and nutritional security, especially for those who consume and depend on corn the most, statistical models can be used to help predict the ideal condition of parameters, understand the influence of pre-treatment on such parameters, and obtain the best design for future actions.

This thesis consists of 3 chapters: 1) a review article (already published); 2) an article on the results of the application of metaproteomics in the study of microbial dynamics (already submitted); and 3) an article about the application of starter strains and pre-treatment of maize before fermentation (under submission). This research was carried out with the following objectives in mind:

Main objective

Improve the production and safety of corn paste using metaproteomics as a fast and efficient tool for studying the optimization of the fermentation process.

Specific objectives

Recreate traditional maize fermentation and monitor fermentative parameters and the microbial count load of relevant food microorganisms.

Apply metaproteomics to fermented maize paste samples and observe the profile and functionality of microbial communities.

Select and apply starter strains predicted as ideal by metaproteomics and perform mathematical modeling adjusted to the kinetics of fermentation parameters to obtain the best fermentation conditions.

CAPITULO 1

Artigo de revisão publicado na revista

Trends in Food Science and Technology

Titulo

Metaproteomics as a tool to optimize the maize fermentation process

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2 METAPROTEOMICS AS A TOOL TO OPTIMIZE THE MAIZE FERMENTATION PROCESS

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Abstract

Background: Maize dough is a fermentation product widely consumed in most African and Central American countries. Functional properties such as prebiotics, probiotics and nutraceuticals of this dough have been widely documented.

However, it is still artisanal and seasonal, without quality assurance and concise shelf-life; the difficulties in processing and conservation force people to discontinue consumption. It is known that this dough forms an indispensable constituent in the daily life of the people in these countries.

Scope and approach: This review shows how metaproteomics data applied to studying the microbiota of fermented maize dough give better insights for optimizing the formulation process. And aim to help rescue and revaluate fermented maize dough in these people.

Key findings and conclusions: Omics are indeed indispensable tools and are increasingly being applied to fermented foods study. Using metaproteomic approaches became easier to make crucial decisions around the formulation, conservation, and shelf life of those products, offering results with a broad spectrum of applications in the fermentation process to optimize it.

Keywords: Maize, fermentation, proteomics, optimization, processing, conservation.

Highlights

Traditional maize dough is an essential source of functional foods, especially in developing countries.

Metaproteomic is a promising omic technology to optimize the process of fermentation of maize dough in all technical aspects and safety.

Optimizing food processes means having safe food and the possibility of industrialization and, therefore, food security for the people who depend on these foods.

2.1 Introduction

Plant-based products are studied and launched on the market frequently, and fermented maize products with their associated functional properties have rich potential as an ingredient in this line. The processes used to obtain maize dough do not involve destruction or harmful transformations, they are natural and free from the addition of chemicals, and can therefore be considered a clean label (Carcelli et al., 2020; S. Park & Kim, 2021). Food research has been affected by a rising and well-established segment of consumers who prefer clean-label meals and foods made with plant-based ingredients (vegetarian, vegan, and flexitarian). They are buyers motivated by moral and environmental concerns as well as the alleged health advantages (prevention of allergy and intolerance problems associated with products of animal origin) (Aschemann-Witzel et al., 2019b; Goswami & Ram, 2017; Hemler & Hu, 2019). Products made from traditionally fermented corn could be termed clean label, plant-based, and have a consistent philosophy of little processing, and with recognized and natural ingredients. In addition, they do lack any potentially harmful elements (Aschemann-Witzel et al., 2019a).

Although corn is the most produced crop globally, more than 80% of it is used for animal feed. Industries use roughly 15% of starch for applications like the manufacturing of bioethanol (Astolfi et al., 2020) or beer production (Romero-Medina et al., 2020). The food industry uses corn as an additive to formulate breakfast cereals in bakeries, cookies, pasta, fats, baby food, pet food, and condiments (Park & Kim, 2021). Corn starch is also used to obtain biodegradable films (Gomez-Aldapa et al., 2020), with antimicrobial function, combined with metals (nanocomponents) or vegetable extracts (polyphenols) (Chen et al., 2020; JHA, 2020; Kumar et al., 2020). .

Nutritionally, corn is an excellent source of carbohydrates since over 70% of its composition is starch (Sjöö & Nilsson, 2017). Lysine and tryptophan are amino acids less commonly found in most types of corn. Unsaturated fatty acids, tocopherol, and antioxidants are abundant in corn germ oil (Which participate in neural and colon protection). Corn coating comprises fibers (Arabinoxylans and β–glucan) complexed with minerals such as phosphorus and magnesium (Achi & Asamudo, 2019; Ibirinde et al., 2020).

It is thought that putting corn through fermentation will boost its nutritional content across the board and get rid of any potential antinutritional factors. Additionally, it enables the acquisition of notable functional features. However, the greatest challenge in traditionally preserving the quality of fermented products is the difficulty in finding a fermentation procedure that ensures a quality product with a long shelf life (Chaves-López et al., 2020; Ogodo et al., 2017).

Spontaneous fermentation processes that produce most traditional foods are still not controlled. The metabolic activity of a complex microbiota succession is a crucial criterion for predicting, optimizing, and managing the formation of flavor compounds and monitoring fermented foods' quality, productivity, and safety (Wu et al., 2021). Indeed, preserving traditional methods contributes to protecting the hidden value of local diversity, especially in developing countries. However, exploring the potential of conventional products in industrial processes is a way to ensure these foods' safety and food security for these people (Galimberti et al., 2021).

Food Control demands efficient, high speed, high productivity detection and traceability of foodborne diseases, and the traditional method of identification by separation can no longer meet the market demand (Balkir et al., 2021). The complexity and dynamic changes of the microbial community make it difficult to understand the processes of spontaneous fermentation, especially when non-recognized or non-culturable microorganisms are involved (Chen, 2022).

Omics-based detection responds to this trend and transforms conventional approaches to food research into cutting-edge approaches that incorporate bioinformatics and next-generation sequencing (NGS). Without the requirement for microbial culture, the omics approach efficiently detects foodborne pathogens using methods applied to the food matrix (Chen et al., 2017b) (Figure 1). Omics tools offer the opportunity to characterize and trace traditional fermented foods, preserve the potential of autochthonous microbial consortia, and access difficult-to-reproduce metabolic pathways (Galimberti et al., 2021).

In this review, we show how the application of proteomics can go beyond the control of food safety and help to obtain data to build an ideal fermentation scheme, more efficient and effective in predicting the components needed to optimize the formulation of corn dough.

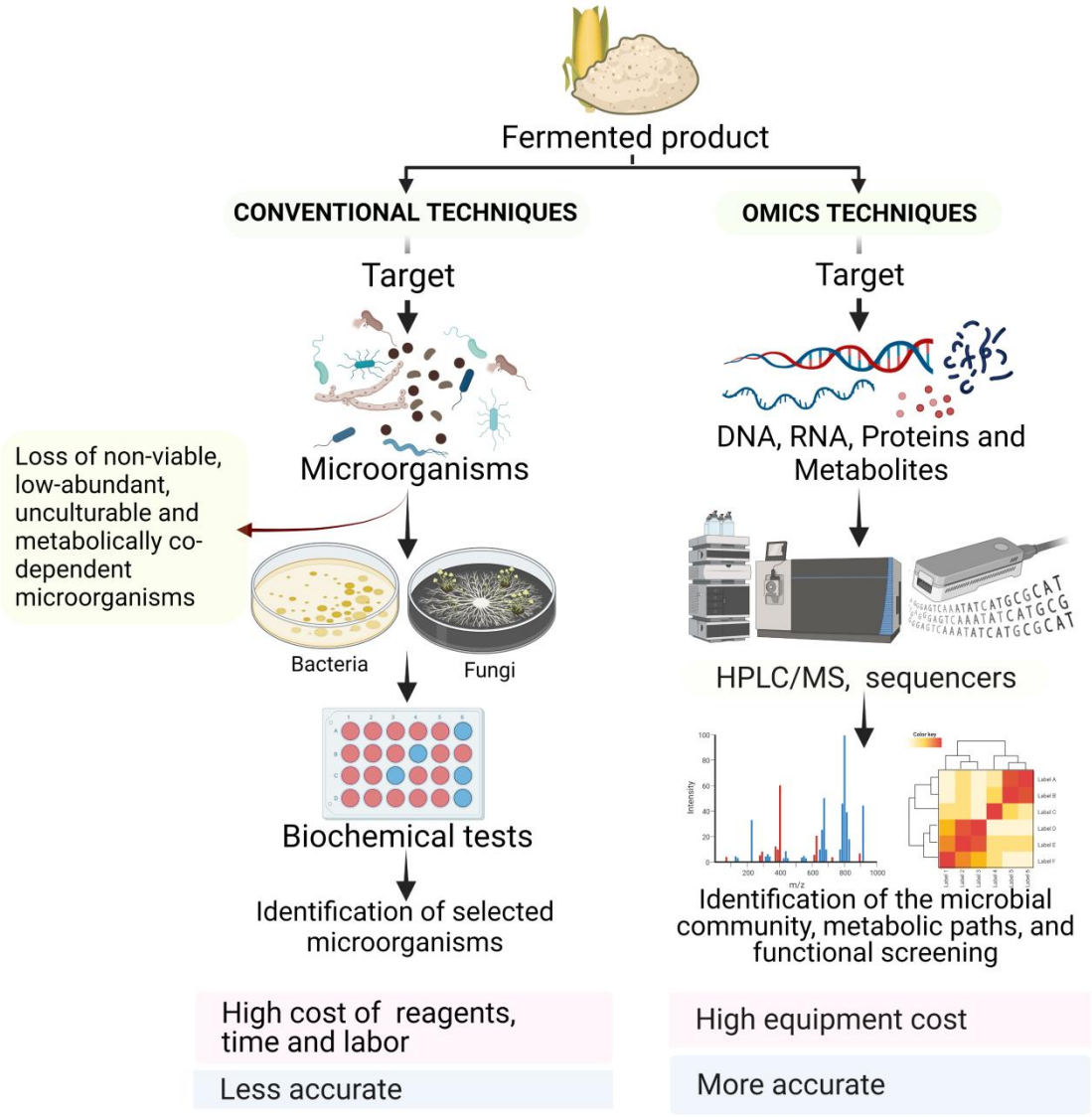

Figure 1. Conventional culture versus metaomics in microbiota analysis.

2.2 Maize fermented dough

It is a pasty and sourdough, with a humidity of about 35%. Corn is the base cereal for the formulation, but other grains like sorghum and millet have already been reported. Since ancient times, maize has been used to make dough. Its preparation is prepared using regional maize types and conventional techniques, but it has not yet undergone widespread industrialization (Chaves-López et al., 2020; Dawlal et al., 2017; Wacoo et al., 2019).

The cleaned corn kernels without bran are fermented for 3 to 7 days and are then ground to form a thick dough or fermented with the bran and then ground and strained to

separate the bran from the starch. The preparation steps and name differ depending on the region where it is prepared. The dough can be called Mbila (Mozambique, South Africa), Ogi and kenkey (Nigeria and Ghana), akamu (Benin) and pozol (Mexico), among other designations. Maize dough is usually used in the preparation of porridges, drinks, pastries, as dough for cakes and various sweets, in addition to accompanying a variety of vegetables, meats, and sauces when cooked, roasted, or fried in multiple ways (Assohoun et al., 2013; Hanvi et al., 2021; Jespersen et al., 1994).

2.2.1 Lactic fermentation process

In the artisanal model, lactic fermentation is done spontaneously, microorganisms are allowed to arise, populate, and make changes to the matrix. The metabolic pathways for metabolized hexoses are heterofermentative (strict or optional) and homofermentative one (Giraffa, 2004). In heterofermentative fermentation, several microorganisms are likely to grow; when the anaerobiosis media is established, just groups of lactic acid bacteria and fungi remain (Gänzle, 2015). Lactic acid bacteria (LAB) are expressive competitors against other groups, as they quickly use abundant sugars and produce an accumulation of lactic and acetic acid. These acids cause the medium's pH to drop, increasing the acidity, which inhibits or limits the growth of bacteria that are less acid-tolerant (Chaves-López et al., 2020). However, suppose there is no reduction in pH; otherwise, inhibited microorganisms may grow, resulting in colonization by bacteria and mesophilic fungi, which more of them are known to be pathogenic, toxin-producing, or spoilage, culminating in a product with an unpleasant and possibly unsuitable for consumption (Giraffa, 2004).

Pyruvate is the primary hydrolysis product of hexoses, although the routes by which it is produced depends on the microbiota and the environment in the medium. Lactate is created when there are too many hexoses in the medium or when anaerobic conditions are created. Pyruvate takes H^+ ions from NADH in lactate to replenish NAD^+ cofactors (Gänzle, 2015). Aside from ethanol produced at the start of fermentation, other byproducts of LABs fermentation include metabolites such as H_2O_2 produced during aerobic development, diacetyl created from surplus citrate-derived pyruvate, and antimicrobial metabolic products (Soro-Yao et al., 2014).

In food fermentation, the natural microflora isn't always effective, manageable, or predictable. Multiple mechanisms allow for microbial interactions in mixed cultures, and these interactions can have positive, neutral, or negative consequences on the fitness of the strains involved (Chaves-López et al., 2020).

In this case, commercial starter cultures for direct inoculation into food have always been recommended for their reliability, performance, safety, and convenience (Giraffa, 2004).

2.2.2 Maize fermentation and functional transformation

Like other cereals, the fermentation of corn is considered one way to increase the nutritional composition, reduce contaminants and obtain functional properties (Achi & Asamudo, 2019; Schwan & Ramos, 2019). Since LABs are known to withstand the physiological hurdless given by the gastrointestinal tract and colonize it, the fact that they ferment corn has many benefits (Ogodo et al., 2017).

In fermented corn, LAB is regularly associated with members of the genera Lactobacillus, Pediococcus, Leuconostoc Enterococcus, Lactococcus, and Weissella. Many species of these genera have been designated as GRAS (generally recognized as safe) by the Food and Drug Administration (FDA) (Chaves-López et al., 2020). Lactic acid bacteria usually found in corn fermentation, generally share the same metabolic and physiological characteristics and are associated with probiotic functional properties (Frias et al., 2017). Significant differences among fermented and non-fermented maize dough relative to the texture (lighter), hardness (25% less, cohesiveness (3 times more), viscosity, and gumminess (increased), were observed (Qi et al., 2019; Reyes et al., 2018; Yang et al., 2016). Significant release of anthocyanins in fermented maize with antioxidant activity was also observed (Romero-Medina et al., 2020).

Organic acids like acetic, propionic, and butyric are produced by LAB, and, it improve consumers welfare and regulate gut environmental conditions. Acetic acid is used in the brain and muscles to generate other molecules and is a precursor to lipids in the liver. While butyric acid in the gut, serves as a source of energy in the colon and a precursor to glutamate and other amino acids, propionic acid contributes in gluconeogenesis in the liver (Chugh et al., 2020; Comerlato et al., 2020; Slizewska & Chlebicz-Wójcik, 2020;

Tseng & Wu, 2019). Lactic acid, on the other hand, reduces the medium's pH, suppressing the growth of other microorganisms in the intestinal environment.

Fermentation of corn with four combinations of different commercial starter cultures *(Lactococcus lactis* CECT 539 *and Pediococcus acidilactici* NRRL B-5627*, Lactobacillus Plantarum* CECT 220) resulted in effective control of *Listeria monocytogenes* (Amado et al., 2012). And probiotic starter cultures have been shown to provide an additional functional effect to fermented corn products, as reduction of aflatoxin, enhance of folate content (Achi & Asamudo, 2019; Greppi et al., 2017; Wacoo et al., 2019). Lactobacillus and bifidobacteria produce folate and riboflavin, which activate the immune system by creating a ligand for the MR1 MHC class 1 molecule that presents an antigen to invariant T cells (MAIT), associated with the intestinal mucosa (Lee & Hase, 2014).

Maize fermentation can make the starchy matrix slightly retrograde, which gives it prebiotic functionality. Studies with fermented corn-resistant starch have shown that it has a better performance in protecting against intestinal diseases and reducing the glycemic index when compared to fiber (Jiang et al., 2020; Sjöö & Nilsson, 2017). Prebiotics are a carbon source for probiotics, they activate the immune system to infection resistance and enhance absorption of minerals and the production of vitamin K and B (9, 12) in the intestine and also, may lower serum cholesterol and prevent carcinogenic tumors (Rawi et al., 2020; Walsh et al., 2020; Wan et al., 2019). Choosing a starter to carry out the fermentation is essential regardless of the purpose. Starters such as LAB or fungi are the most suitable for corn fermentation, especially for quick lactic acid obtention, inhibition of mesophilic microorganisms, more stability, and pleasing aroma. The preparation of many traditional fermented foods and beverages uses naturally prepared starter cultures to improve the fermentation process (Frias et al., 2017; Goulas et al., 2021). On the other hand, businesses enhance the processes of food fermentation by injecting previously examined lab-grown cultures (Qi et al., 2019). This cultivated section of the microorganisms, however, only makes up a small portion of the overall estimated bacterial and fungal biodiversity in a medium, as was established by the entire genome sequencing carried out decades ago (Filippis et al., 2017; Song et al., 2021). The omics are a new approach to microorganism analysis that uses genome, transcriptome, proteome, and metabolome technics and data (Wilson & Piel, 2013). When omics technologies are applied to fermented foods studies, they may provide

valuable insights into predicting functional proprieties and pathways of all cultivated and non-cultivated strains (Rizo et al., 2020; Villarreal et al., 2018).

2.3 Omics and the fermentation process

Several aspects are involved in fermentation, highlighting the influence of the substrate, the expected product, and the microorganisms used (Qi et al., 2019). Omics technology provides exciting details, and in such a short time, without the need for cultivation of fermented products microbiota, and, proteomics is the most used tool (Chen et al., 2017a). Omics are genetic study techniques that provide phylogenetic, biochemical, and functional data of microorganisms with the difference of not requiring previous cultivation of microorganisms as in traditional methods (Figure 2). They provide much broader and more relevant results for studying these microorganisms in the environment in which they are inserted.

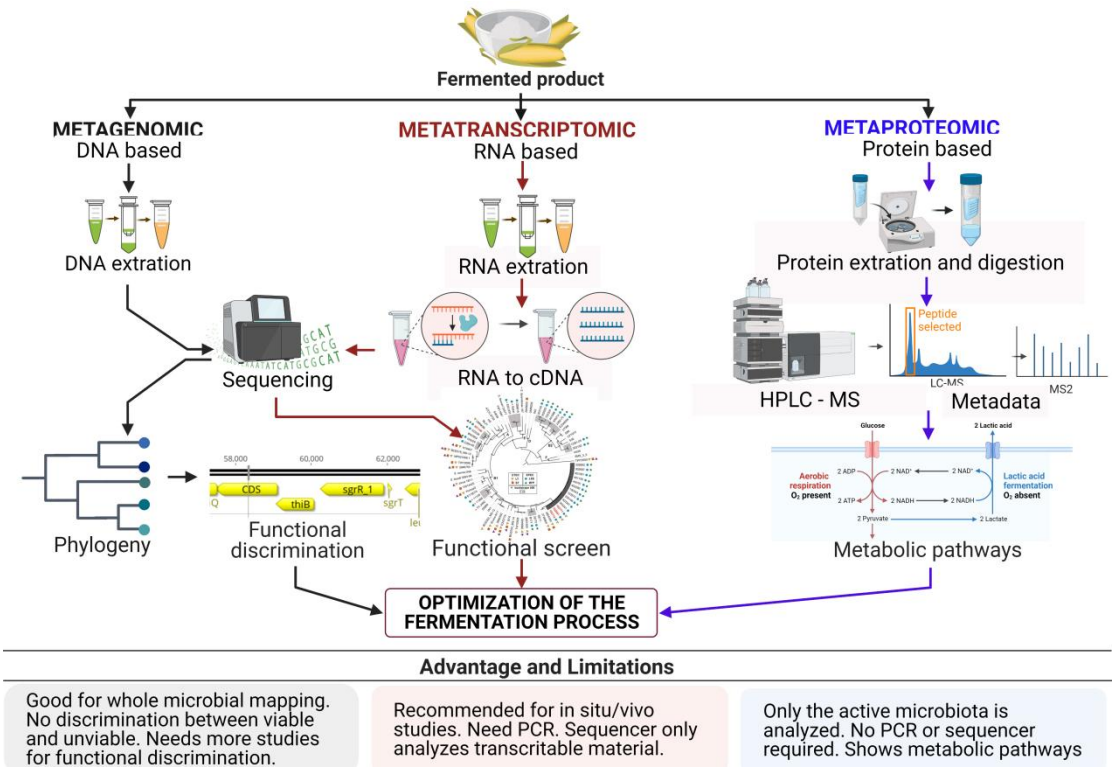

Figure 2. Schematic representation of the main omic tools and the possibility of using them to characterize the microbiota and optimize the fermentation process.

2.3.1 Metagenomics

Metagenomics is an omic tool that analyzes the DNA of a mixed population of organisms through the sequencing or not of food samples (Shokralla et al., 2012). It uses bioinformatic screening methods, to identify new biocatalysts and biosynthetic genes. For identification, clones from metagenomic libraries are screened to find similar phenotypes, modifications, or specific enzyme functions. Specific reporter genes can also be used to identify functional biocatalysts (Balkir et al., 2021), or functional screens that do not require prior knowledge of gene sequences are used to identify new biochemical mechanisms (Wilson and Piel 2013). The correlation between phylogeny and functional attributes depends on factors including trait complexity. Still, the degree of correlation suggests that it may be helpful to predict functions encoded in an organism's genome based on functions encoded in closely related genomes (Langille et al., 2013). For instance, the phylogenetic analysis is performed by comparing communities of a genetic database, where it observes the similarity and determines whether the microorganism present and its dynamics are. The result of the metagenomic analysis is usually a screen or metagenomic library, (figure 2) that indicates the communities that were founded in a food sample (Balkir et al., 2021).

Another techniques that uses DNA approach with unrequirement of the cultivation of microorganisms, used for food analysis are: The heterogeneity-length polymerase chain reaction (LH-PCR); amplified fragment length polymorphism (AFLP); internal transcript spacer (ITS) restriction analysis, terminal restriction fragment length polymorphism (T-RFLP), single-stranded conformational polymorphism (SSCP); ribosomal amplified DNA restriction analysis (ARDRA); DNA amplified polymorphism (RAPD); ribosomal intergenic spacer analysis (RISA); denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE), and next-generation sequencing (NGS) by the HiSeq Illumina System pyrosequencing (Villarreal et al., 2018).

The most applied ways in foods for the microbiological study are: (a) amplification (by PCR) of the 16S, 18S, or 26S rRNA genes to estimate the microbial diversity; (b) digestion of genetic material and cloning into expression vectors; and or (c) direct sequencing of the food sample. DGGE is one of the most used techniques to identify

microorganisms during fermentation processes, food spoilage, and food safety, as it offers an economic advantage (Villarreal et al., 2018; Wilson & Piel, 2013).

Despite the high cost, NGS has an advantage over DGGE, as many sequences can be analyzed quickly using technologies such as Illumina and Roche 454 pyrosequencing, which are more efficient than DGGE detection (Castro-López et al., 2021).

Furthermore, the NGS technique can show the dominant populations and predict those responsible for the organoleptic characteristics in food matrices, as well as the composition of the microbiota during the different stages of processing and manufacturing, also allowing the detection of difficult species cultivation (Bilal et al., 2018). is the need to analyze more than one sample to carry out the study and, often, follow up on changes in a certain food that further aggravates the costs of the NGS technique.

Through a metagenomic approach, DGGE (V3 region of the 16S rRNA gene) recognized yeasts, fungi, and bacteria at the same time from a spontaneous fermentation sample of pozol (Mexican food made from fermented corn) (Villarreal et al., 2018).

Metagenomics can go beyond community identification or phylogenetic listing and allow a functional prediction of microbial communities. The possibility of using this omic to allow the optimization of the fermentation process would require additional studies for the functional discrimination of the microbiota (figure 2). However, because metagenomics uses DNA as a target for analysis, it is difficult to discriminate which microorganisms are or are not cultivable/unviable, leading to an inaccurate extrapolation of the real microbial activity. In addition, it would be necessary to carry out periodic analyzes with several points from the beginning to the end of the fermentation to get an idea of the dynamics of the fermentative microbiota. Another omics approach that is more valuable for a functional prediction and to show in the real time, the role of each one in the community revealing information regarding ongoing protein expression and post-transductional modifications is metatransriptomic.

2.3.2 Metatranscriptomics

It is a crucial omic for the functional characterization of microorganisms, with the advantage of being carried out in situ and analyzing RNA. It assumes that the RNA in the fermentation environment indicates the state of the physiological activity of specific

microorganisms and that this may indicate their role in the modification of the food matrix under analysis. The main difference between metatranscriptomics and metagenomics (figure 2) is the possibility of applicability in situ. After collecting samples, total RNA is extracted, residual DNA is removed, and mRNA is amplified. As mRNA is easy to degrade, it is necessary to reverse the RNA to cDNA and then sequence the cDNA using the sequencing platform (Ding et al., 2020; Weckx et al., 2011) The result of metatranscriptomics can be an amplicon library or a functional screen according to interest or both.

After sequencing, the phylogenetic investigation of communities by reconstruction of unobserved states (PICRSt) approach is used, using an extended ancestral state reconstruction algorithm to predict genes present. It combines families to estimate the composite metagenome. It is a computational approach to predicting functional composition using marker gene data and a reference genome database. Using information from the 16S RNA gene, PICRSt accurately predicts the abundance of gene families in communities associated with specific environmental conditions with quantifiable uncertainty (Langille et al., 2013) in composting, metatranscriptomics showed the changing trends of the bacterial community, and, through PICRUSt, functional changes of bioreactor-fermented composts and a traditional way were predicted (Ding et al., 2020).

Barcode multiplex pyrosequencing of a V1-V3 region of the 16S gene (rRNA) tagged with sample-specific barcodes for multiplex identifiers was employed to assess the bacterial community profile and showed diversity, richness, and also variation depending on the various types of kimchi (fermented vegetables) analyzed. The correlation between ingredients and manufacturing processes was also explained (Park et al., 2012). The next-generation sequencing (Illumina MiSeq Platform) as a tool to identify microorganism communities without culturing them was used to observe microbial dynamics during cupuassu fermentation. The role of pulp concentration for bacterial populations and biochemical transformations was seen, and the consortium of microorganisms essential for forming desirable flavor precursor compounds was also predicted (Ramos et al., 2020).

The Metatranscriptome analysis is limited concerning how gene expression and regulation in a microbiome influence the functional response between the microbial community and the product modification through its interactions. Also, metagenomic

analysis has limitations in revealing information regarding ongoing protein expression and post-traduction modification. in this case, the metaproteomic approach can and is currently the most suitable for studying fermentation environments in foods.

2.3.3 Metaproteomics

The dynamic character of metaproteomics allows not only to identify the proteins being expressed at a given moment but also to quantify them and observe their post-transductional modifications, with the advantage of allowing the identification of occasional variations in this content under the action of different stimuli (Goulas et al., 2021; Rizo et al., 2020). Due to the excellent chemical diversity of proteins and their interconnectivity in signaling complexes and networks, proteomic analysis has advantages and a more significant number of variables concerning genomics and transcriptome analysis. The study of proteins requires analytical tools with high selectivity, resolution, and sensitivity (Braga Emidio et al., 2015).

Two-dimensional electrophoresis (2D) is the most commonly used method of applying an electric field to separate proteins. First, according to the isoelectric point, in a pH gradient gel, the proteins migrate until reaching their isoelectric point. Subsequently, by molecular volume, consisting of an SDS-polyacrylamide gel, SDS detergent-coated proteins migrate through the interwoven network formed by the acrylamide, separated by their molecular mass (Han & Wang, 2008).

The 2D analysis allows a preliminary view of the protein content expressed in the sample (by observing the expression variations when comparing the position and intensity of the bands obtained). Electrophoresis can be one of the steps of mass spectrometry, where the sample is pre-fractionated (Yang et al., 2020). In this, identified bands are excised, digested, and then analyzed by a mass spectrometer to determine and characterize proteins.

Another approach dispenses pre-fractionation, known as "shotgun proteomics," based on the digestion of the full protein extract and separating the peptides by multidimensional liquid chromatography. In the shotgun strategy, there are two approaches: the "bottom-up" type, which is the most used, in which there is protein hydrolysis by peptidases and then analysis by chromatography coupled to a mass spectrometer. The other approach is top-down, which, in turn, does not use the previous

digestion of the proteins to be analyzed. Despite appearing to be a less complex strategy, it presents technical barriers and a higher cost (Balkir et al., 2021).

2.4 Metaproteomics as a tool for optimizing the maize dough fermentation process

Metaproteomics is one of the omics tools widely applied in fermented foods to understand microbial interactions, fermentative kinetics, and metabolic profiles during the fermentation process and assess the functional diversity of microbial communities (Yang et al., 2020). It has already been used to screen for contaminants and toxins that conventional methods cannot detect and even predict a food's shelf life (Zhang et al., 2019). The main hypotheses raised in proteomics studies are that knowing the protein profile and its functions (signals, structural, regulation, or enzymes), it is possible to design probable metabolic routes, where it is possible to visualize what happens in the fermentation medium (Ferrocino & Cocolin, 2017; Filippis et al., 2017).

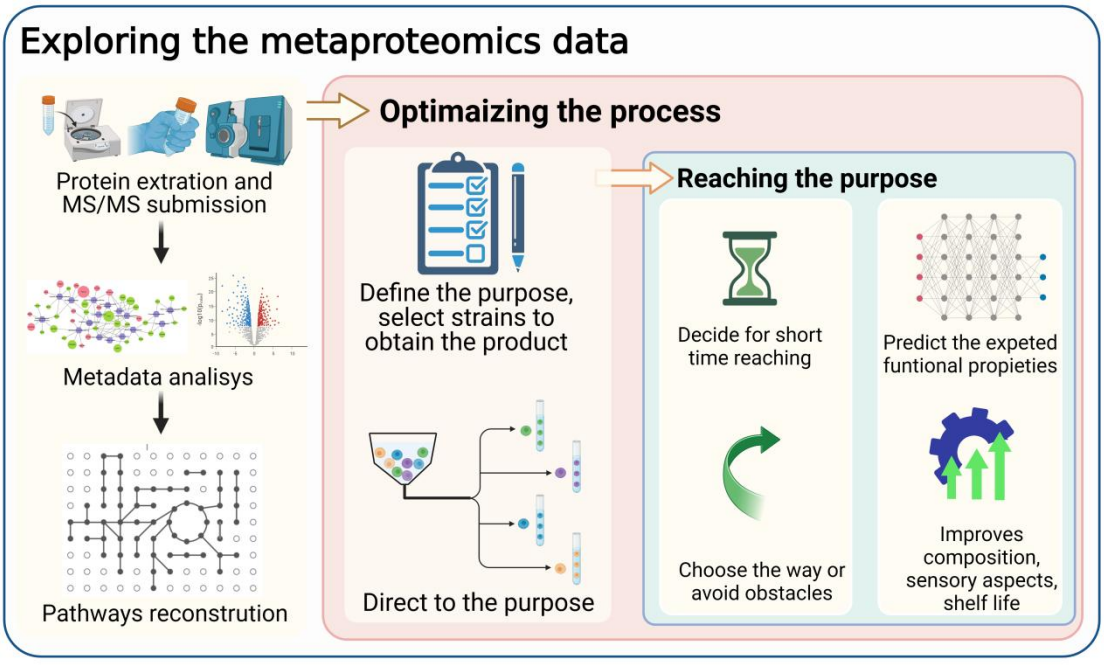

Figure 3 Metaproteomic approach for optimization of maize dough, an example of a way out

An optimal process is one in which a product is obtained in a short time at low cost, with all sensory, nutritional, functional, and sanitary aspects attended, and which

guarantees better and greater conservation of the product (Goyal et al., 2022; Schwan & Ramos, 2019) For example (figure 3), knowing the metabolic pathways can allow choosing the starter strain and:

- ✓ Induce this to produce specific metabolites of interest.
- ✓ Know the ideal carbon sources to supplement the medium and enhance the performance of the starter.
- ✓ Choose intermediate strains and know the right moment to introduce them.
- ✓ Introduce strains that end the fermentation to use metabolites that can harm the quality and preservation of the products.
- ✓ Identify strains and their particularities without requiring biochemical tests.
- ✓ Establish an antimicrobial action by identifying possible links between starter and pathogenic strains.
- ✓ Find possible biological markers of the fermentation process and new routes, and thus predict and avoid inhibitory pathways or enhance pathways of interest.

Proteomic analysis revealed how the modifications caused by reactive oxygen species (ROS) impaired the fermentation capacity of the *Saccharomyces cerevisiae* during fermentation. A decrease in the activity of pyruvate decarboxylase (a critical enzyme involved in the production of ethanol) and also the enzymes affected site was acessed (Eknikom et al., 2022).

In another study *Trichoderma reesei* was cultured on different carbon sources (cellulose, sophorose, and glucose) to understand the molecular basis of lignocellulose-degrading enzyme induction. Comparing the transcriptome (RNAseq) and secretome (2-D DIGE) was revealed that proteins with non-catalytic functions secreted in unusual carbon sources induced cellulase enzyme production (Dos Santos Castro et al., 2014) Metaproteomics tools identified proteins and enzymes that facilitate the use of the substrate and the microbiota dynamics in the fermentation of corn dough (pozol), as well as the origin of the secretory activity and the time of metabolic activity of the *Lactobacillus* and *Aspergillus* strains linked to this process (Cárdenas et al., 2014).

2.5 Final considerations

As already described, spontaneous fermentation of corn paste can occur at different times (24h to 15 days) depending on the corn variety used, the degree of maturation and/or the ambient temperature Another problem is that there is no guarantee that in this spontaneous fermentation, other pathogenic microorganisms cannot grow and reduce the quality and acceptance of the product. In corn, the starch is the primary substrate, more likely fermented with lactic acid bacteria or yeast, whose carbon source is glucose and not specifically starch. In this way, more than selecting ideal starters, suitable enzyme production pathways are needed to easier and faster reach the purpose of the process. Like enhance enzymatic inhibitory metabolites or delay the inhibition. In addition, fix how to conduct the obtention of functional proprieties like probiotic or mycotoxin suppress ways. Biochemical transformations that determine sensory aspects in corn fermentation can be understood using metaproteomics and predicted in models that are subsequently applied for optimization.

Handling the right tools in fermentation can mean minimal processing, no addition of preservatives, and reduction of production time, obtaining functional and sensory properties of interest, and extending the fermented maize's shelf-life dough.

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CAPITULO 2

Manuscrito submetido para a revista Food Chemistry

Título

Metaproteomics revealing microbial diversity and activity in the spontaneous fermentation of maize dough

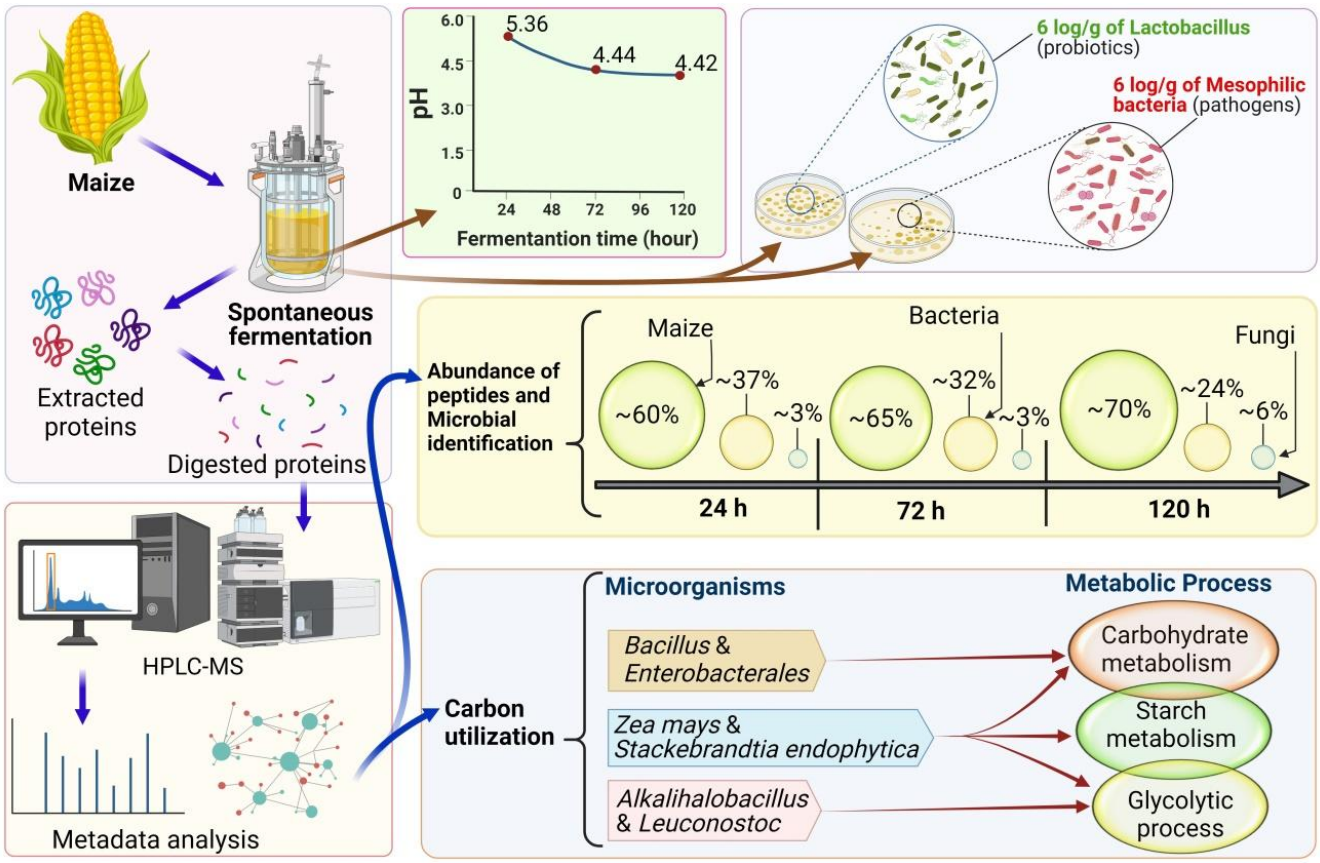
4 Metaproteomics revealing microbial diversity and activity in the spontaneous fermentation of maize dough

Abstract

Maize was spontaneously fermented for five days to study the profile of microbial communities, and parameters and microbial count values were determined. A metaproteomic study was performed on the maize dough. A wide range of microbial groupings have been found. The pH was gradually reduced (5.36, 4.44, and 4.42 after 24, 72, and 120 hours, respectively), while lactic acid levels increased (0.03, 0.2, and 0.31% after 24, 72, and 120 hours, respectively). The lactic acid bacteria count (179 x 10^6 CFU/g) and mesophiles count (213 x 10^6 CFU/g) were also high. The fermentation environment was dominated by Actinobacteria, Proteobacteria, and Firmicutes, which grew and coexisted until the end. There were representatives from all pathogenic proteobacteria classes. Several actinobacteria phylum were connected to the maize matrix during the starch-degradation process. Within the first 24 hours, fermentable sugar sources are required to maximize productivity and ensure the microbiological safety of maize dough. Metaproteomics is a strong method for examining microbial dynamics and succession in fermented foods in a comprehensive, quick, and accurate manner.

Key words: proteomics, fermentation of corn, biological processes, microbial succession, optimization

Graphical abstract



Source: elaborated by the author

4.1 Introduction

Cereal grains are an essential component of the human diet, nourishing millions of people with nearly half of the world's caloric requirements and 37% of the protein intake (Ranum et al., 2014; FAO, 2022). Three cereal grains have been described as the essential food for humankind: wheat (*Triticum aestivum*), rice (*Oryza sativa*), and maize (*Zea mays*) accounting for 94% of cereal grain consumption (Ranum et al., 2014). Among them, maize has had a global harvested area of more than 234 million hectares in the 2020 season, with an estimated production of more than 1.4×10^9 t (FAO, 2022). In South America, Brazil is the largest maize producer, with a harvested area of 18 million hectares and a productivity of 1.0×10^8 t, being the third-biggest producer in the 2020 season after the United States and China (FAO, 2022). Maize can be consumed processed or unprocessed, amounting to 18.5 kg/capita/year (Erenstein et al., 2022).

Maize is a large part of the diet in many countries worldwide, especially in developing countries from Latin America and Africa. When fermented, mixed cultures of microorganisms develop, consisting mainly of lactic acid bacteria (LAB), yeast, molds, acetic acid bacteria, and *Bacillus* spp., which contribute to the sensory characteristics, nutritional value, and shelf life of maize products (Chaves-López et al., 2020) . The impact of the activities of this complex microbiota on the development and attributes of the maize product varies according to the communities involved. It may positively influence the safety profile, such as detoxifying phytates and reducing mycotoxins, negatively promote rapid putrefaction, make sensory aspects unpleasant, or even cause severe contamination (Chaves-López et al., 2020). The spontaneous fermentation environment makes it challenging to control its performance parameters because of different strains' behavior (Phiri, 2019) , which is why most kinetic models generally seek to reduce the modeling error by using isolated, selected, and previously studied strains with a substrate more available and controllable (Astolfi et al., 2020; Phiri, 2019). Therefore, microbial communities' specific effects and dynamics throughout the maize fermentation are unknown.

To optimize maize fermentation to formulate the maize dough, several studies with different strains or substrate treatments would have to be carried out, as well as testing distint models to find the most suitable. This whole process around optimization would demand a high cost in time, resources, and computer knowledge (Gbashi et al., 2020). Currently, there is a tendency to associate analysis techniques that can shorten the path, reduce the cost, and increase the accuracy of research involving microorganisms in a complementary or even integral way (Filippis et al., 2017) . These techniques do not require microbial cultivation and use RNA, DNA, or proteins of the samples to obtain the complete profile of the microbial community. Microbial DNA extraction and amplification by PCR using specific primers allowed the analysis of the microbial profile of fermented maize to obtain silage (Jiang et al., 2020). Another DNA analysis by MiSeq for 16S rRNA gene sequencing and operational taxonomic units (OTUs) was carried out to study the bacterial community succession (Xie et al., 2017); and to identify critical bacterial populations for selecting the most suitable strains to produce high-quality dajiang and other fermented products (Xie et al., 2019).

Omics techniques such as metagenomics and metaproteomic are widely used in environmental and medical studies. They are also applicable to foods area, mainly in

fermentation processes, with the advantage of providing a profile and the functional screen of the microbiota without cultivation requirements and with more accuracy. In metagenomics, the DNA analysis shows profile of active, inactive, cultivable, and non-cultivable microorganisms (Filippis et al., 2017) . In contrast, metaproteomic analyzes the peptides and proteins in a sample. It can display the profile of active and functional microbiota, and any activity and functionality of the fermentation substrate's peptides and proteins (enzymes) (Bahule et al., 2022). Studies reported metaproteomic as a powerful tool that uses data from all proteins in the samples and provides direct evidence of diversity and functional structure and microbial activity between the microbiota in niches of any environment and the possibility of using metaproteomic data to optimize the process of maize fermentation (Bahule et al., 2022).

This study is the first to use metaproteomics as an analysis method to examine the microbial profile of maize's spontaneous fermentation process. A database of proteins and peptides will be made available for future identification. It will provide as a foundation for decision-making regarding the optimization and industrialisation of fermentation processes as well as for a better comprehension of the microbial profile.

The proteome profile, dynamics, and interactions of the microbiota are examined in this study using metaproteomic data from spontaneously fermented maize. Finding and choosing qualities of the optimal starter for maize fermentation and dough formulation, which can be inexpensive and allow the preservation of the nutritious and hygienic features, would be beneficial.

4.2 **Material and methods**

The present research used spontaneously fermented maize dough to conduct the metaproteomic analysis. The experiment and analysis were accomplished in the Laboratory of Biotechnology Processes, Federal University of Pará (LABIOTEC, UFPA, Brazil). White maize (Real brand), broken and without bran, with specifications to the seller as for (mixed *group, peeled subgroup, type 1, white class*), was purchased at the local market of Belem (PA, Brazil) to be used as substrate throughout the experimental test. The maize grain (1500g) was duly washed with distilled water and weighed. A 5 L container, previously washed and disinfected with 70% iodinated alcohol, was submitted to sterilization with UV light in a laminar flow cabinet for 15 minutes. Then, the maize grain and distilled water (2.5 L) at room temperature (30 ±

1.5ºC) were added to the fermentation vessel (Poliester, 5 L capacity, with cover for anaerbiose) and placed in the fermentation room. The experiment was performed in triplicate.

4.2.1 Fermentation

The fermentation consisted of spontaneous fermentation, where microorganisms grew freely on the substrate at room temperature (30 ± 1.5ºC) for 120 hours in an isolated room and in facultative aerobic conditions. The fermentation vessels remained closed during fermentation and were opened every 24 hours to check the fermentation performance parameters and for sample collection. During the process, the fermentation temperature (with a laser thermometer), the pH (digital pH meter), acidity (by titration with NaOH, at 0.1mol/L), and starch content (Fehling A and B) were measured. On the last day of the fermentation, the alcohol content (alcoholometer) was also measured. The bacterial counting (mesophiles and *Lactobacillus* spp.) was measured after 21 days of fermentation. MRS Agar medium from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) was used for *Lactobacillus* and Bacterial Agar Couting medium for mesophiles.

4.3 *Metaproteomic analysis*

4.3.1 Protein extraction

Protein isolation was performed according to Wang et al. (2006), with modifications described in Trindade et al. (2021). Briefly, 3 g of maize dough collected at 24, 72, and 120h of the fermentation were macerated using liquid nitrogen. Then, 9.0 mL of extraction buffer (0.85 molLexp. -1 sucrose, 0.1 molLexp. -1Tris-HCl (pH 8.0), 2% (w/v) sodium dodecyl sulfate (SDS), one molLexp. -1 phenyl-methylsulfonyl fluoride, and 2% (w/v) polyvinylpolypyrrolidone) were added to the macerated dough. After that, three molLexp. -1 of Protease Inhibitor Cocktail Powder (Sigma-Aldrich, St. Louis, MO, USA) and 70 molLexp. -1 of dithiothreitol (DTT) were added, incubated at room temperature for 10 minutes and then sonicated five times (30 s for the event and 30 s intervals). Each fractioned sample was performed in 8 microtubes and was homogenized individually with 700 µL saturated phenol (pH 8.0). The phenolic phase of each aliquot was collected and washed twice by centrifugation (14,000 x g for 7 min at 4 °C) to remove any residue of SDS or the aqueous phase. The phenolic phase was

collected, and proteins were precipitated by incubating the samples with 800 µL 0.1 molLexp. -1 ammonium acetate (prepared in absolute methanol at -20 °C) at -80 °C overnight. Then the samples were centrifuged (14,000 x g for 7 min at four °C) and discarded the supernatant. The remaining protein pellet was washed with 1.5 mL of 80 % (v/v) ice-cold acetone and ethanol. Finally, the resulting pellet was dried in a vacuum concentrator for 7 min. Protein extracts were solubilized by adding 100 µL of 0.1 % RapiGest™ Surfactant (Waters, Milford, MA, USA) and stored at -80 °C until protein digestion. The protein concentration was estimated using the Qubit 3.0 Fluorometer (Invitrogen, Waltham, MA, USA).

4.3.2 Protein digestion and sample desalting

Fifty micrograms of proteins were quantified per sample. The preparation for digestion involved protein reduction with DTT 5 molLexp. -1 and incubation at 56 °C for 25 min, followed by alkylation with iodoacetamide (IAA) 14 molLexp. -1 and incubation at room temperature for 30 min. Residual IAA was removed by adding DTT (5 molLexp. -1) and incubating at room temperature for 15 min, followed by the addition of CaCl2 (1 molLexp. -1) and treatment with trypsin (20 ng µL-1) at 37 °C for 20 h. Subsequently, trifluoroacetic acid was added to a final concentration of 0.4 % to stop the enzymatic reaction. The samples were incubated at 37 °C for 90 min, then centrifuged at 14,000 x g for 130 min at four °C. Finally, the supernatant was transferred to the appropriate vials. The pH solution was adjusted to 10 with 1 N ammonium hydroxide for effective trapping on the first-dimension column of the ultra-performance liquid chromatography (UPLC).

4.3.3 Protein identification and bioinformatics

The protein identification and quantification were performed according to the methodology described in Nascimento et al. (2022a) with minor modifications. For peptide identification, a nanoACQUITY UPLC® (Waters) was used. A total of 50 µg of the digested proteins were analyzed with five replicates. The first dimension used a 5 µm XBridge BEH130 C18 (300 µm × 50 mm) and a Symmetry C18 5 µm (180 µm × 20 mm) trapping column at a flow rate of 2000 µL min^{-1}. Then the samples were passed through a 1.7 µm BEH130 C18 1.8 µm (100 µm × 100 mm) analytical column at a flow rate of 400 µL min^{-1}. The samples were separated into five fractions with a gradient of 10.8, 14.0, 16.7, 20.4, and 65.0 % acetonitrile. This liquid chromatography was coupled

to a NanoLock ESI-Q-ToF SYNAPT G2-S (Waters) mass spectrometer, configured for data acquisition ranging from 50 to 2000 Da in MS^E mode at a scan rate of 0.5 s and an interscan delay 2.0 of 0.1 s. The raw data was processed using the Progenesis QI software v.2.0 (Waters), as reported by Nascimento et al. (Nascimento et al., 2022b). The results were exported to the Scaffold Proteome Software v.4.6.1 (Proteome Software, Portland, OR) for validation and visualization (Searle, 2010). Peptides were accepted when the calculated false discovery rate was lower than 6.6 % and a threshold of 3.3 % for the identified proteins. The functional analysis of the assigned proteins was obtained from the software Unipept v.4.0 using default parameters (Mesuere et al., 2015) (https://unipept.ugent.be/datasets, accessed on 15 December 2022) to get Gene Ontology (GO) terms, Enzyme Commission (EC) numbers, and taxonomic classification. The differences between samples were estimated based on a functional or taxonomic composition using Unipept (Verschaffelt et al., 2021).

4.4 Data Analysis

The statistical tests were conducted using the R software (R Core Team 2018; https:// www.R-project.org), considering statistical significance at $p < 0.05$.

4.5 Result and Discussion

4.5.1 Performance parameters of the maize fermentation process

The results showed a typical tendency of lactic fermentation, in which the temperature and pH started high and were reduced with the fermentation time. The titratable acidity (corrected for lactic acid) tended to increase (from 0.03 to 0.3 %) from 24 to 120 hours, respectively (Table 1). An average pH value (of 4.42) was checked at the end of fermentation. The fermentable sugars showed an irregular behavior, without any clear trend (Table 1), but with relatively high values, which was somewhat contrasting, configuring a fermentation that can be unstable. The results showed that both lactic acid and mesophilic bacteria were present in high densities and a ratio of 1:1.2 (Table 1). No alcohol was detected in the product (Table 1), which confirms a typically lactic fermentation. (Rizo et al., 2021) studies with fermented maize noted that the main changes in both the substrate and the microbiota occurred in the first 9 hours of

fermentation and with microorganisms increasing in correlation with the drop in pH and the decrease in carbohydrate content.

Table 1: Maize fermentation Parameters collected during the recreation of traditional spontaneous fermentation

Fermentation parameters	Fermentation time (hour)			
	Unit	24	72	120
T	ºC	30.9	30.0	28.2
pH	--	5.36	4.44	4.42
L.ac	%	0.03	0.20	0.31
FS	mg/L	2709.3	3690.0	2115.6
MBC	CFU/g	--	--	213 x 10^6
LBC	CFU/g	--	--	179 x10^6
Alcohol content	m/v	--	--	0

T: Temperature; pH: Hydrogenion potential; FS: Fermentable sugars; L.ac: Lactic acid; MBC: Mesophilic bacterial count; LBC: Lactobacillus bacterial count (MRS Agar).
Source: elaborated by the author

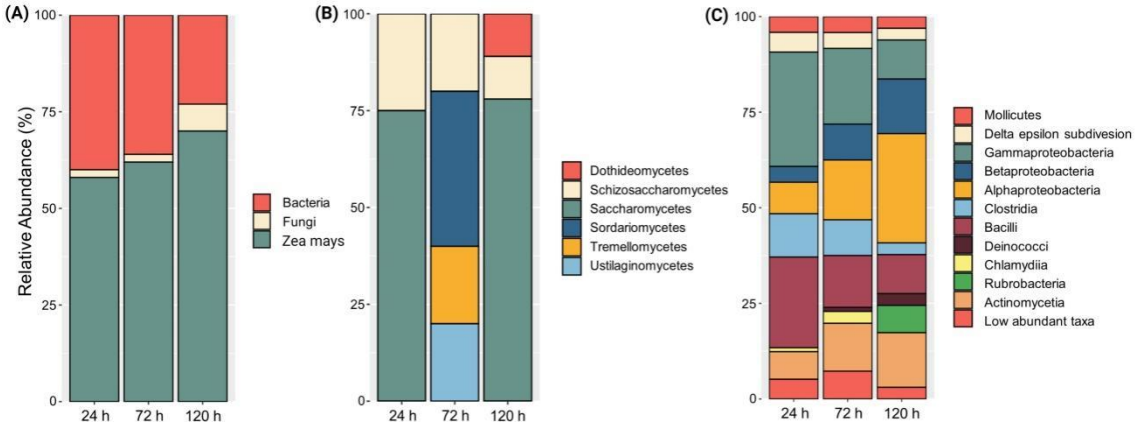
Despite observing a typical evolution of fermentation, the average value of lactic acid (0.3%) and pH (4.42) represents a concern since lactic acid was not in sufficiently high amounts to reduce the pH to safer values (3.8 - 4.5) taken as reference the acidity guidelines for fermented milk (MAPA, I.N.46/OUT/2007, Brazil, 2005). The pH value (4.42) was within the recommended range, although very close to the minimum safe limit for fermented food (Wu et al., 2021).

The high density detected and in similar proportions of lactic acid bacteria (GRAS) and mesophilic bacteria (which include potential pathogens) in the fermented product leaves doubts about the classification of this food as safe or not, based only on the count of GRAS or mesophiles present in this food, (Ray, 2004; Zommiti et al., 2020). Therefore, methods such as omics, complementary or substitutes for counting, are necessary to provide more accurate results (Bahule et al., 2022).

4.5.2 Metaproteomic of the maize dough fermentation process

During the maize fermentation process, a total of 53977 peptides were recovered by metaproteomic analysis, distributed as follows by different sampling times: 15639 (24

h), 21630 (72 h), and 16708 (in 120 h) (Dataset S1). Identification showed that > 60% of these peptides were associated with maize, the remainder with the microbiota, ~6% with fungi, and 34% with bacteria (Fig. 1). Metaproteomic, in addition to correctly identifying and quantifying the peptides in the samples, also made it possible to discriminate them according to the different sources of origin (Fig. 2A, 2B, and 2C). Data referring to the relative abundance of peptides from the identified microbial classes (Fig. 1 B and C) showed that the spontaneous fermentation environment of maize at room temperature is optimal for the growth of a diversity of microorganisms judging by the communities of bacteria and fungi found. The identified microbiota belongs to several phyla, mostly being anaerobes of intestinal origin and from environments such as soil (mud, marine sediments) and compost (Fig. 1). Despite the fungus, the bacteria expressed better in the maize lactic fermentation medium. Indeed, several studies have already reported the probable reduction (Mokoena et al., 2006), detoxification (Dawlal et al., 2017; Hashemi et al., 2021) or even suppression (Okeke et al., 2021) of fungal growth in environments of lactic acid fermentation, depending on the species present as well as their density.



Source: elaborated by the author

Figure 1: Relative abundance of total peptides (A), fungal phyla (B), and bacterial phyla (C) in fermented maize dough

4.5.3 Dynamics and succession of the microbiota during fermentation

The evolution of fermentation showed a dynamic of microbiota succession, and the details are shown in Fig. 2. In the first 24 hours of fermentation, the most abundant peptides were assigned to Firmicutes, Proteobacteria, and Actinobacteria. Until 120 hours of fermentation, these phyla dominated, with a slight reduction in Firmicutes and an increase in Proteobacteria, the latter having all classes (alpha, beta, gamma, delta, and epsilon) represented (Fig. 2). The dynamics of fungal evolution were less vigorous, but they also increased over fermentation time.

Proteobacteria and Firmicutes are very important phyla in fermentation studies, as they include most groups of pathogens, but also other groups (Bacilli) known as “generally recognize as safe” (GRAS) and for their probiotic functionality (Sharma & Tripathi, 2019). The phylum Firmicutes are commonly associated with Bacteroidetes in the human gut flora and as endophytic microbiota of plants, in the human, both phyla constitute the intestinal flora in proportionally balanced quantities, where most Bacteroidetes than firmicutes presume a healthy intestine (Tseng & Hu, 2018).

Our results showed that in this fermentation, the phylum Firmicutes was in more significant proportion than Bacteroidetes, and the latter was not identified at the beginning of fermentation (24h). It is not known if the fermentation behavior can be translated similarly when this food is consumed; moreover, no association between these phyla was analyzed in this study.

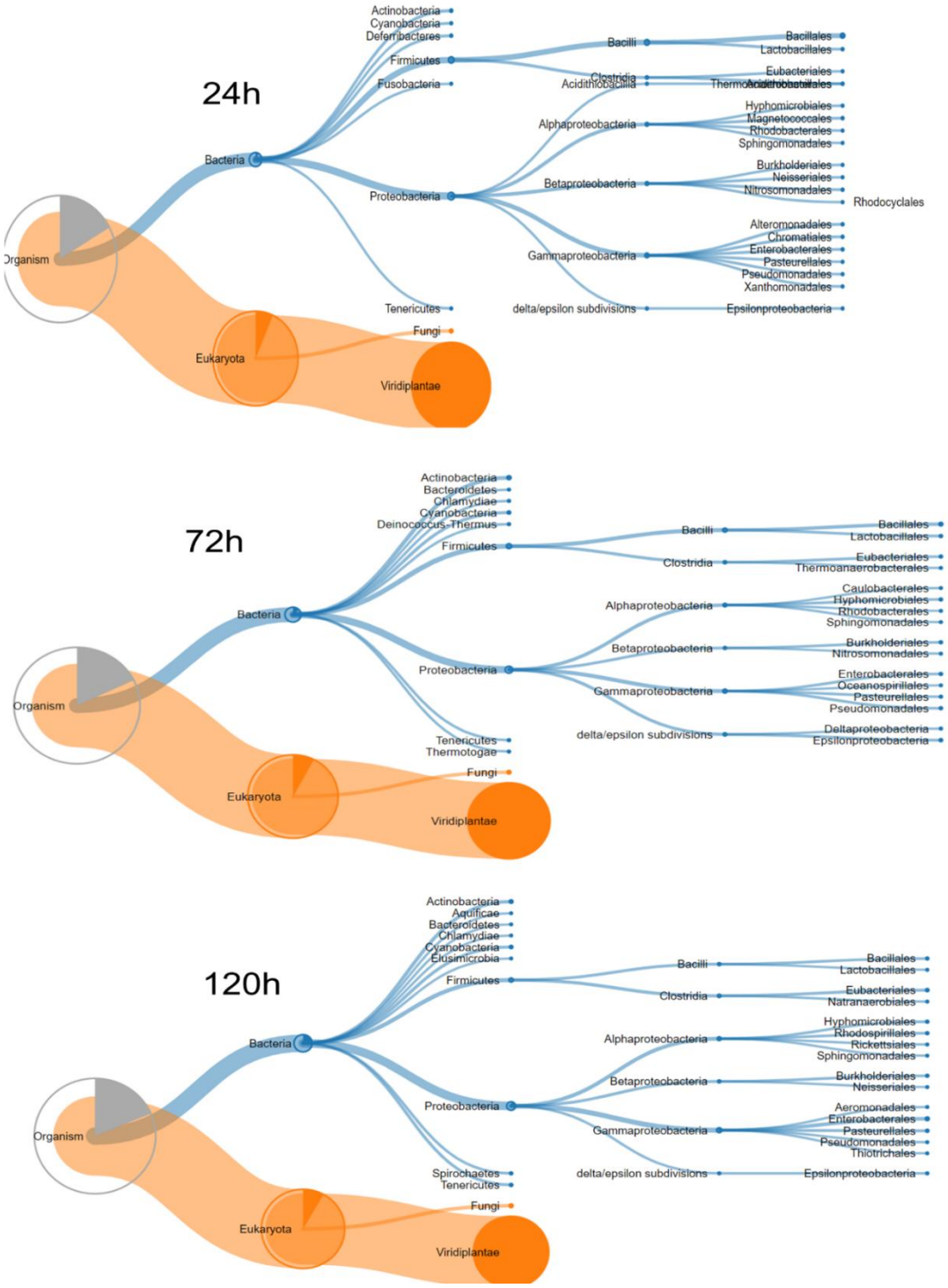


Figure 2. Peptide-based dendrogram constructed by Unipept 4.0 using all peptides obtained from fermented maize with significant protein matches. The phylogenetic tree was constructed using the lowest common ancestor (LCA) method. The circles' sizes refer to the peptide abundance for each taxonomic level.

4.5.4 Microbiota profile of fermented maize dough

About 120 genera of bacteria and 14 fungi were identified and are presented in Table 2. Some bacterial genera identified here include significant pathogens associated with foodborne diseases, such as Escherichia, Salmonella, Enterobacter, Yersinia, Pseudomonas, Helicobacter, Clostridium, Staphylococci and Campylobacter (Bintsis, 2017). Other genera, most considered safe (GRAS) (Chibueze Izah et al., 2016; Marco et al., 2021) were also identified, among which the Bacillus, Streptococcus, and Lactobacillus. In turn, the detected fungi integrate genera with potentially pathogenic as well as non-pathogenic representatives; however, even if in considerably smaller amounts, the presence of genera such as Aspergillus, Candida, and Mucor is a crucial safety alert sign (Ghosh et al., 2021) , mainly due to the risk of toxin production, for example by *Aspergillus* spp. (Garrido-Bazan et al., 2018; Stagnati et al., 2020).

Other genera of microorganisms identified include phytopathogens (e.g., Giberrella; Alternaria, Xanthomonas) or humans and animals (e.g., Leptospira, Mycobacterium, Encephalitozoon, and Clostridioides), with antibiotic activity (Streptomyces) and still others that can be found living freely in the soil and water (e.g., Nitrosomonas, Methylobacterium, Rhodobacter, Paracoccus, and Thiobacillus) (Khanna & Kraft, 2021; Ray, 2004).

The conjugation of the presence of the genera of lactic acid bacteria, together with the count obtained from these, confirm the potential functional probiotic effect that maize dough can have (Table 1, 2) (Ray, 2004). Controversially, the high count of mesophilic bacteria suggests a risk of contamination in products consumed uncooked, given that most of the identified genera contain potentially pathogenic strains (Table 2). The risk of food contamination becomes greater when considering that some fermented maize products do not undergo any heat treatment after fermentation (Chaves-López et al., 2020) . As an example, we have the *Ogi maize dough*, typically consumed in Nigeria, Ghana, and surrounding countries, which is even given to children as the first food from 6 months of age, consumed by adding warm water to the fermented dough (Okeke et al., 2021) . Kwete is a lactic drink commonly consumed at all ages; even though the flour undergoes pre-rosted, spontaneous fermentation is the last preparation stage before consumption (Wacoo et al., 2019).

Table 2. Microbial genera identified in fermented maize dough based on the peptide sequence using the Unipept 4.0 software.

Source: elaborated by the author

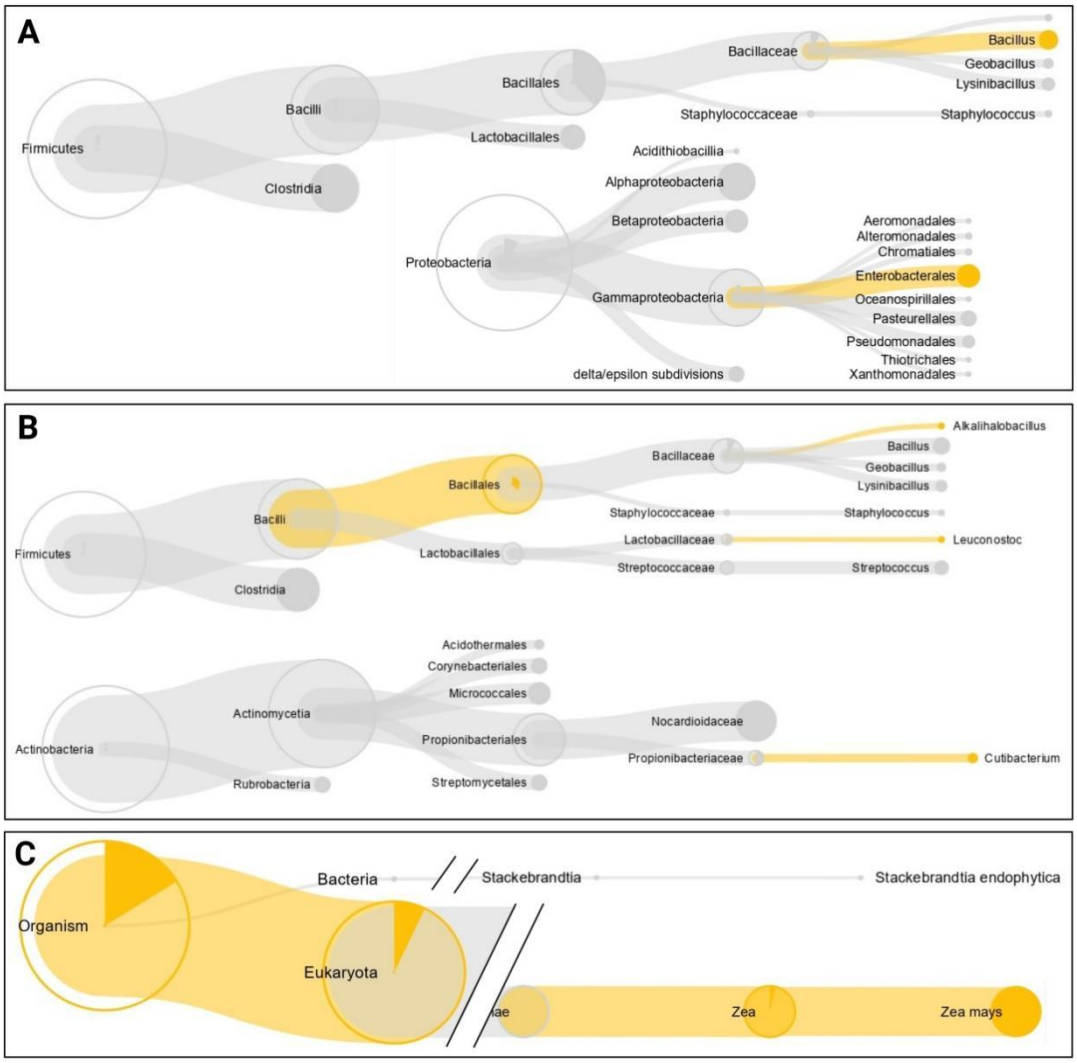
Genera of Bacteria			Genera of Fungi
Acidithiobacillus	Enterobacter	Lysinibacillus	Alternaria
Acidothermus	Erythrobacter	Magnetococcus	Ashbya
Acidovorax	Escherichia	Magnetospirillum	Aspergillus
Actinobacillus	Francisella	Mannheimia	Candida
Aeromonas	Fusobacterium	Mesoplasma	Encephalitozoon
Akkermansia	Geobacillus	Methylocella	Gibberella
Alicyclobacillus	Geobacter	Methylococcus	Kluyveromyces
Alkalilimnicola	Gloeobacter	Methylorubrum	Mucor
Anaplasma	Gramella	Methylovorus	Ogataea
Anoxybacillus	Haemophilus	Moorella	Saccharomyces
Aquifex	Helicobacter	Mycobacterium	Schizosaccharomyces
Azoarcus	Idiomarina	Mycoplasma	Ustilago
Azobacteroides	Klebsiella	Natranaerobius	Wickerhamomyces
Azorhizobium	Koribacter	Neisseria	Zygosaccharomyces
Bacillus	Lactobacillus	Nitratiruptor	
Bacteroides	Leifsonia	Nitrosomonas	
Bartonella	Leptospira	Nocardia	
Baumannia	Leuconostoc	Nocardioides	
Bradyrhizobium	Listeria	Novosphingobium	
Buchnera	Xanthomonas	Ochrobactrum	
Burkholderia	Yersinia	Paenarthrobacter	
Caldanaerobacter	Salmonella	Paraburkholderia	
Caldicellulosiruptor	Shewanella	Paraburkholderia	
Campylobacter	Sodalis	Paracoccus	
Carboxydothermus	Spiroplasma	Pasteurella	
Caulobacter	Staphylococcus	Pelobacter	
Chlamydia	Stenotrophomonas	Pelotomaculum	
Chromohalobacter	Streptococcus	Persephonella	
Clostridioides	Streptomyces	Prochlorococcus	
Clostridium	Symbiobacterium	Protochlamydia	
Corynebacterium	Synechococcus	Pseudomonas	
Crocosphaera	Synechocystis	Pseudothermotoga	
Cronobacter	Syntrophobacter	Psychromonas	
Cryptococcus	Syntrophomonas	Ralstonia	
Cutibacterium	Thermotoga	Renibacterium	
Dechloromonas	Thermus	Rhizobium	
Denitrovibrio	Thiobacillus	Rhodobacter	
Desulfococcus	Tolumonas	Rhodopseudomonas	
Edwardsiella	Treponema	Rubrobacter	
Wolinella	Verminephrobacter	Wolbachia	

Source: elaborated by the author

4.5.5 The biological process of carbohydrate degradation in the fermentation process of maize

Fig. 3 shows the association between the biological process and the individuals responsible for it. Bacteria played an essential role in the breakdown, and use of various carbohydrates and glucose (Fig. 3A and B); fungi were not associated with these analyzed processes and probably used these sources in a less expressive way or even other carbon sources.

Among bacteria, the Bacilli classes (genus Leuconostoc and Alkalihalobacillus), Proteobacteria, and Actinobacteria (genus Cutibacterium) were the groups with the most significant participation in the enzymatic activity of degrading glucose and other carbohydrates (Dataset S1). The metabolic process of starch breakdown was associated only with the substrate matrix (maize grain) and Actinobacteria (Fig. 3 C). The real contribution of that before referred groups and others identified as well, with lesser expression to maize fermentation and enzymatic activity need to be studied more deeply.



Source: elaborated by the author constructed by Unipept 4.0

Figure 3. Origin of the main proteins (enzymes) that participate in the different processes of carbohydrate metabolism: (A) Carbohydrate metabolic process. (B) Glycolytic process and (C) Carbohydrate metabolic, glycolytic and starch biosynthetic process.

Our results showed that the maize matrix exerted the greatest enzymatic activity in breaking down starch into glucose and in breaking down glucose itself. Generally, the changes that occur in maize fermentation, such as the appearance of organic acids and phenolic compounds, increase in protein, reduction of phytates, and digestibility, are always attributed to the metabolic action of microorganisms (Jiang et al., 2020; Romero-Medina et al., 2020; Wu et al., 2021).

The enzymatic activity of the maize matrix detected in this study had yet to be reported in any other research on maize fermentation to produce edible dough. Therefore, a

careful analysis is necessary to interpret the results obtained by metaproteomic. Some studies that analyze the enzymatic activity of whole grains that are germinated before consumption report a group of enzymes and other products different from those observed in this study. (Hernández-Becerra et al., 2020; Garcia-Ortiz et al., 2023) consider that there are enzymes in the endosperm of the grains (concentrated in aleurone). However, germination is what mobilizes for the emergence of the activity of enzymes that hydrolyze macromolecules such as starch and compounds such as β-glucans and phytic acid. In most cereals, the maximum activity starts from the fourth day of germination.

The probable explanation for the enzymatic activity of the grain may be its association with Actinobacteria. This group of bacteria is known to be endophytes (they live symbiotically in plant tissue), may or may not be saprophytic (degrade dead tissue) and have antibiotic action (mostly antifungal). They produce various enzymes (including amylolytic) intra and extracellularly (Gohain et al 2020; Correia et al., 2019). Another explanation is that some endosperm enzymes that break glycolytic bonds are independent of the germination process, and their activation is water in the grain-soaking process. However, it is worth mentioning that this work will not deepen the enzymatic activity analysis. Therefore, a specific future study is necessary.

4.5.6 Moving towards optimized fermentation, what to consider?

In this study, were analyzed the microbial profile and the carbon sources associated with each group of microorganisms to understand their dynamics and thus decide the succession we are interested in developing. Metaproteomics revealed that spontaneous fermentation of maize could be an optimal environment for the development of functional foods, but at the same time, dangerous. Therefore, measures to optimize it are the appropriate way out. Based on the results obtained in this study, some aspects that eventually influence fermentation's failure and success were identified; below are the most relevant and likely strategies that can be implemented for optimizing and formulating safe and functional food dough.

Very high microbial load and diversity

Maize proved to be an excellent substrate for diversified microbiota. Foods obtained by spontaneous fermentation and without treatment to reduce the microbial load will certainly allow growth if they are active, representing a potential danger or even a source of functional properties, so there is a need to apply a method of suppression of growth, reduction, or elimination of undesirable microorganisms mainly at the beginning of fermentation (24h) or before.

A slight decrease in pH

Studies highlight the expressiveness of amylolytic lactic acid bacteria (ALAB) in breaking down starch and their agility in using sugars when abundant, which makes these bacteria excellent competitors and suppressors of others by producing organic acids and increasing the acidity of the medium (Gänzle, 2015; Ogodo et al., 2017). In this study, it was verified that both the presence of *Lactobacillus* and the high availability of fermentable sugars did not result in the expressiveness of *Lactobacillus*, and the pH reduction was slow to the fact that the breakdown of starch was associated with Actinobacteria and the maize matrix. It is necessary to guarantee the availability of sugars in the first 24 hours and, at the same time, allow the fermenting strains to be present in the ideal amount to reduce the pH to safe levels before 48 hours of fermentation. Strategically inoculating a starter after 24 hours of fermentation is a practical solution; allows rapid suppression of mesophiles and little competition as starch and sugars are available for transformation into lactic acid.

Microbial diversity and, in significantly significant quantities

The spontaneous fermentation of maize is not a recent subject. Despite the divergence of opinions, there is agreement on the probiotic potential and, simultaneously, the contaminating potential resulting from this process, as was proved in this study. After all, Bacteroidetes (known as the beneficial majority), Firmicutes (where the primary lactic acid fermenters are found), and Proteobacteria (with a pathogenic and mesophilic majority) had a harmonious coexistence. Suppressing the growth of one group to the detriment of the other is strategically necessary; whose manipulation exercise must include modifying all aspects of spontaneous fermentation. For example, raising or lowering the temperature, strategic addition of fermentable sugars, and timely use of a starter or intermediate strain during fermentation. Adding exogenous sources of fermentable sugars during fermentation would fortify the *Lactobacillus* spp., as they are

avid users of sugars. Under stress, they produce lactic acid to suppress the other groups. 1 – The strain that establishes an acidic environment more quickly in the medium helps to suppress the non-tolerant majority. 2 - Strains resistant to high (above 45°C) or low (below 15°C) temperatures can be an intelligent choice to suppress most mesophiles. 3 - Strains that produce metabolites that suppress other pathogenic strains, but are not harmful to the consumer, can also be a strategic solution.

4.6 Conclusions

Metaproteomic was applied to study the profile of microbial communities in spontaneous fermentation for maize dough production. Bacteria dominated the fermentation medium, and both lactic acid and mesophilic bacteria were at high densities, suggesting that fermented maize dough is a potentially probiotic and microbiologically unsafe food. Although bacteria have participated in critical metabolic processes, such as the carbohydrate metabolic process, and glycolytic processes, the participation of maize proteins was predominant in these processes, in addition to being the only ones to participate in the starch biosynthesis process. The branches of exogenous sources of rapidly fermentable carbohydrates in the first 24h and the inoculation of lactic strains are viable strategies to optimize the production and guarantee the microbial safety of the maize dough. Metaproteomic is indeed a tool for studying the microbial profile, and with this tool, it was possible to carry out a complete study on the fermentative microbiota of maize.

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CAPITULO 3

Manuscrito a ser submetido para a revista

A selecionar

Título

Pediococcus acidilati in maize dough formulation: a metaproteomic approach to better decide the fermentation parameters

6 Pediococcus acidilati in fermentation process of maize for dough formulation: a metaproteomic approach to better decide fermentation parameters

Abstract

The metaproteomic data of the spontaneously fermented maize dough were explored in terms of enzymatic activity and microbiota functionality, and it was observed that an unprecedented enzymatic activity of the substrate would have affected the process of using carbohydrates (starch) and allowed the harmonious growth of several species, both pathogenic and non-pathogenic. This led to the understanding that spontaneous maize fermentation poses a danger and that a strategic starter needs to be used. With the aim of making maize fermentation safer, the performance of the *Pediococcus acidilati* strain was then evaluated, where previously treated corn (T+I) at 121°C for 20 min to eliminate enzymatic interference from the substrate and microbiota was subjected to In a controlled fermentation, untreated corn (nT+I) was also inoculated with the strain. The 120-hour experiment was carried out in triplicate, and the main parameters were measured 24 hours after fermentation. The results showed a strong correlation between the important fermentation parameters (pH and lactic acid) in the T+I compared to the untreated one (nT+I). The fact that the pH of the medium was below 4.5 in the first 24 hours of fermentation reveals the high applicability of this strain and the possibility of reducing processing time to obtain a safer food product. *Pediococcus acidilati* showed excellent performance as a starter strain in maize fermentation; however, for the first time, it was used in maize for the formulation of a food product, so studies on the metabolomics of this process are encouraged.

Keywords: carbohydrate , starch, lactic acid, microbial enzymes, starter

6.1 Introduction

Pediococcus is a lactic acid bacterium isolated from different sources, such as cocoa (Chagas Junior et al., 2022), silage (Fugaban et al., 2022), milk (Dewi et al., 2021), and also found associated with a mushroom (Hermawan et al., 1970). This species was identified in 1970 *and has* since been exhaustively studied and widely applied. Its application is mainly as a probiotic feed additive in broilers and fish (Ferguson et al., 2010; Maniat et al., 2023), where oral supplementation has been shown to modulate intestinal bacterial communities and stimulate some aspects of the nonspecific immune system response. *P. acidilactici* GR-66 can significantly alleviate Cr (VI)-induced inflammation and oxidative stress (Wang, 2023). It was characterized as a bacteriocin producer when strong antimicrobial activity was detected against more than 74 different strains of *Listeria monocytogenes* and 27 different vancomycin-resistant Enterococcus strains, showing its effective antimicrobial action (Fugaban et al., 2022), and also proved to be applicable in studies for the production of L-LA (lactic acid) from softwood hydrolysate, which is considered an inhibitor (Campos et al., 2023). In foods, it was applied in the dry fermentation of sausages, where, when compared to the autochthonous microbial population present in the control lot, it did not negatively modify the physicochemical parameters or the sensorial quality (Ruiz-Moyano et al., 2011). In their study, Abbasiliasi et al. (2017) showed that *P. acidilactici* exhibited properties that suggested its potential application as a probiotic and starter culture in the food industry. *P. acidilactici* DNH16 isolated from dairy foods due to its high tolerance to acidic environments and bile salts (0.3%), indicated by percentage growth of 91.6 and 66.5% in MRS medium containing 0.3% bile salts with a pH of 3, and by presenting 27% α-glucosidase inhibitory activity, was identified with the potential to be used as a probiotic as well as an -glucosidase inhibitor (Fachrial et al., 2023).

In our previous experiment, spontaneous fermentation and subsequent dough formulation were carried out following traditional procedures, and metaproteomic analysis was performed to assess the screen peptides and their functionalities (Bahule et al., 2022) and to select an ideal strain for maize dough fermentation. After deep analyses of the spontaneous fermented process through metaproteomic data, the behavior and functionality of *Pediococcus acidilati* strains were considered applicable starters. The aim of this study was to evaluate the applicability of *Pediococcus acidilati*

as a starter strain in the maize fermentation process through the evaluation of the main parameters of fermentation.

6.2 Materials and methods

The present research was done using spontaneous as control and no-spontaneous fermentation with heat pre-treatments of the maize grain. The LAB used during the experiment was *Pediococcus acidilati MRS 45* from the Laboratory of Biotechnology Processes, Federal University of Pará (LABIOTEC, UFPA, Brazil). For activation of the *Pediococcus acidilati* LAB strain, the MRS broth medium from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) was used, and then MRS agar was used for plate counting.

6.2.1 Strain activation

The microorganism *(P. acidilati*) was activated according to the manual of activation procedures proposed by Chagas Junior (2017), available at LABIOTEC/UFPA. Activation consisted of introducing 200 g of the strain into 100 mL of MRS broth medium in 250 mL of Erlenmeyer, previously autoclaved at 121 ºC for 15 min. The mixture was left in the mixer device for 12 hours at 37 ºC with agitation at 150 rpm (Peters et al., 2019); after that, this mixture was added to 900 mL of sterilized MRS broth, which was kept under agitation (a magnetic stirrer) at 37 to 38.5 ºC. Every 4 hours, 1 mL aliquot were withdrawn, added to 9 mL of peptone water, and then serial dilution was performed for colony counting. One (1) mL of each dilution was seeded by depth in a Petri plate on MRS Agar and then incubated for 48 h for readings. After reaching the required count, the already turbid medium containing the strain was centrifuged for 15 min at 4°C and 14,500 rpm. The supernatant was removed, and the pellet was recovered. After this phase, the LAB concentration was determined by calculating the precipitate weight and counting kinetics. The amount of 4,44g of recovered inoculum, with a count of 19,6.10 log CFU/g, was diluted in 120 mL of water at 37 ºC and then distributed in the six fermentation buckets, after which the corn and then the water were added to the buckets and placed to ferment.

6.2.2 Fermentation of maize grain

The corn grain (1500g) was duly washed with distilled water, weighed, and then reserved. A pre-treatment of corn by autoclaving at 121°C for 20 minutes was also performed. The following treatments were performed:

Table 1: Treatment performed as Pre-treated (T) and non-pre-treated (nT) corn; inoculated (I) and non-inoculated (nI) with *Pediococcus acidilati* strain

Nr	Treatment	Cod
1	Pre-treated corn and inoculum	T+I
2	Untreated corn and inoculum	nT+I
3	Untreated corn without inoculum	nT=nI

The inoculum was the *Pediococcus acidilati* strain referred to before. A 5L recipe, previously washed and disinfected with 70% iodinated alcohol, was submitted to sterilization with UV light in laminar flow for 15 minutes. Then maize grain and distilled water (2.5 L) at room temperature were added to the recipe and placed in the fermentation room. The experiment was performed in triplicate, and data measurement of the main parameters was performed 24 hours after fermentation for five days.

Physic-chemical analysis

The physical-chemical composition of maize corn was analyzed before and after fermentation tests. The grain of the maize was ground in an analytical mill (A11, IKA Staufen, Germany). The analysis was carried out according to the Association of Official Analytical Chemists (AOAC), where moisture content (method 931.04), pH (method 970.21), and total titratable acidity (TTA, method 31.06.06) were measured (Horwitz & Latimer, 2006). Total reducing sugars (TRS) by the 3,5-dinitrosalicylic acid method (Miller, 1959). Protein values were determined by Kjeldahl methodology (1883), lipids by hot extraction in Soxhlet, and Mineral Matter by incineration in the muffle; all analyses were performed in triplicate.

6.2.3 Statistical analysis

The results were submitted to an analysis of variance (ANOVA), and the means were compared by Tukey's test at a 5% significance level. Statistical analyses were performed using Jamovi 2.3.2.1 software. Factors that affect the fermentation of maize grains were studied, and a correlation/regression test was performed with a prior analysis of normality to see the correlation between factors. The ANOVA OF REPEATED measurements was made to see the behavior of the main parameter at different times and with different treatments.

6.3 Results and discussion

Corn fermentation was carried out by observing the criteria of the traditional model, where microorganisms are allowed to grow spontaneously and there is no temperature control. This spontaneous fermentation served as a control to evaluate subsequent fermentation, and then a corn paste was formulated and metaproteomic analysis performed. The relative abundance obtained by the metaproteomic analysis of corn paste in the first 24 hours and at the end of fermentation at 120 hours is illustrated in Figures 1 and 2.

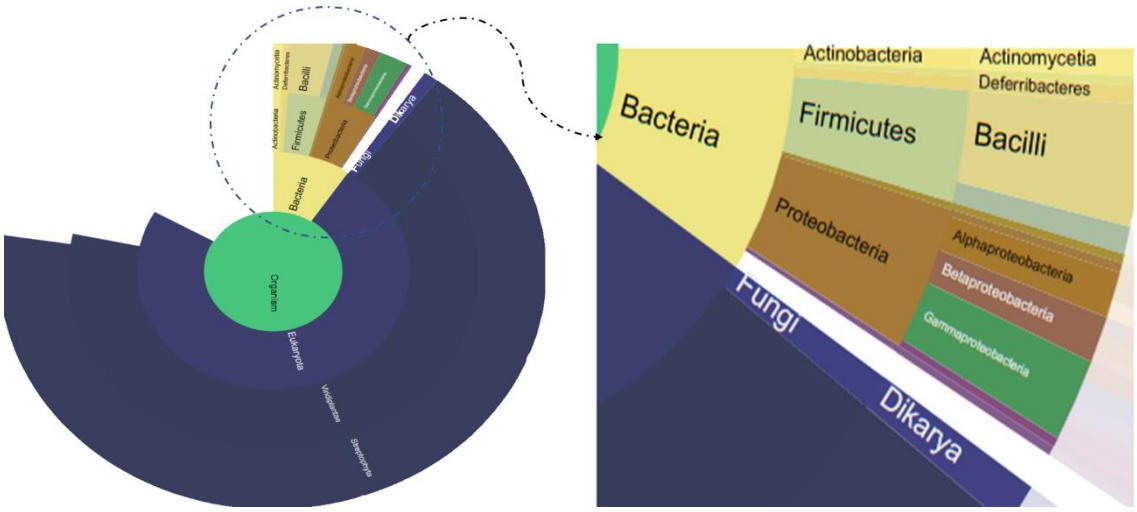

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Figure 1. Diagram illustrating the relative abundance of peptides and the main groups of individuals identified in 24 hours of fermentation.

The relative abundance of microorganisms after 24 hours of fermentation (Figure 1) shows that bacteria dominated the system, with the main focus on firmicutes and

proteobacteria. These mentioned groups are gram-positive and grow in relatively low oxygen environments; however, while the firmicutes (represented by the bacilli) are avid consumers of glucose and producers of lactic acid, the proteobacteria use mostly pyruvate as a carbon source. During the fermentation, at 120 h (Figure 2), the scenario was changing and benefiting the proteobacteria, where in all classes of these pathogenic bacteria there were representatives, and at the same time in firmicutes another group (Clostridia) was becoming evident. Observing the expressive growth of several pathogenic species leads to the understanding that the spontaneity of fermentation represents a danger and that there is a need to optimize the fermentation process (Chaves-López et al., 2020). Therefore, the metaproteomic data had to be explored in an integrated way at the level of the microbe and its functionality.

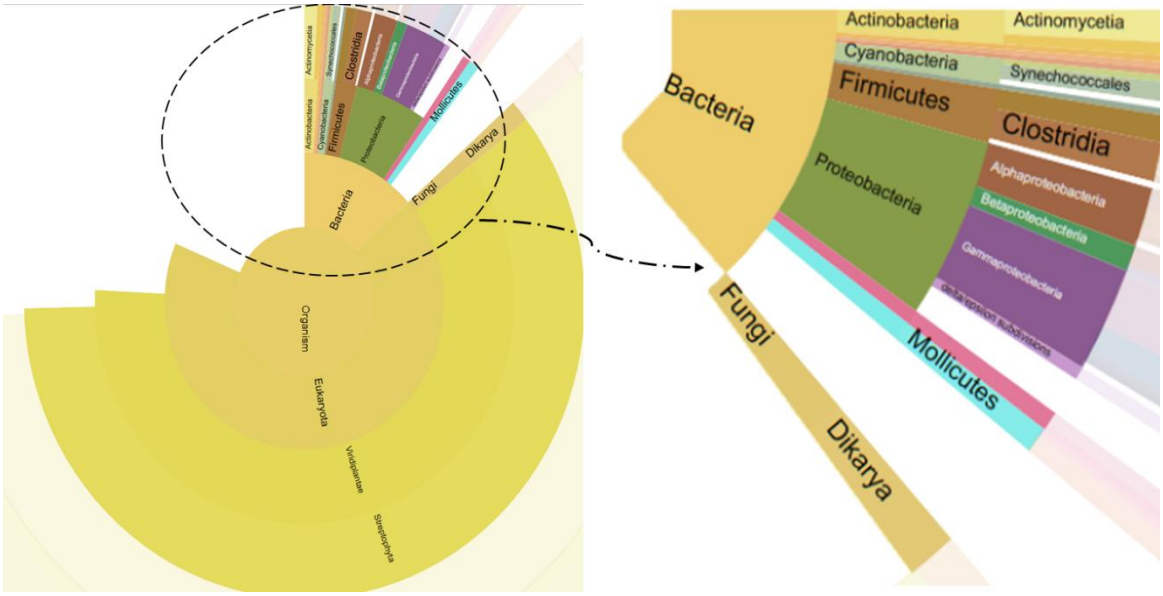

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Figure 2. Diagram illustrating the relative abundance of peptides and the main groups of individuals identified in 120 hours of fermentation

The diagram shown in Figure 3 shows the summary of all enzyme groups identified in the system from the initial 24 hours (306 proteins (58%)) to the end of 120 hours (517 proteins (51%). It can also be seen that transferases (EC:3) have the highest activity, followed by hydrolases (EC:3), and then oxidoreductases (EC:1). Most transferases (ex: pyruvate phosphate dikinase) participate in the Krebs cycle, phosphate pyruvate, or

linking monosaccharides to form sucrose (sucrose synthase); those processes are frequent in plant organisms but rarely occur in microorganisms.

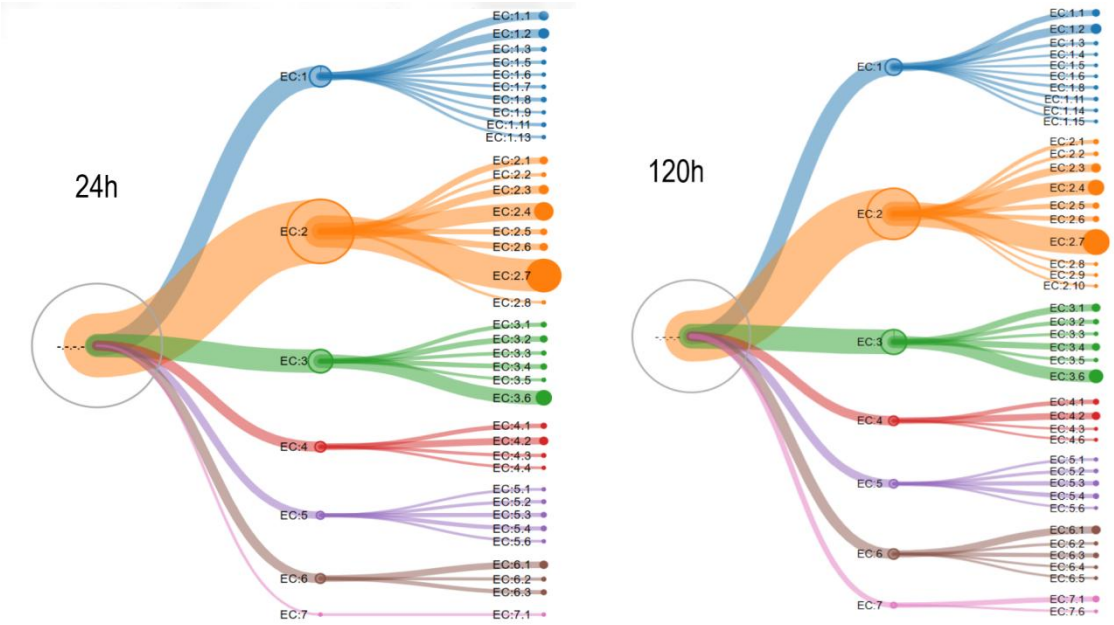

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Figure 3. Illustrative diagram of the expression of enzymes recovered from maize dough, during the beginning (24h) and the end (120h) of fermentation.

The enzymatic groups show that the action that occurred during fermentation was largely due to the substrate (corn grain) and also attributed to microorganisms. Bacteria had the second-highest enzyme activity (Figure 4). Contrary to this study, in most fermentation studies, where the enzymatic activity has always been attributed to microorganisms (Chaves-López et al., 2020; Ogodo et al., 2017), in this study a quite different scenario was verified. No reports from other studies are available, indicating the possibility of the enzymatic action of maize grain during fermentation.

Therefore, here we verify the advantage of the metaproteomic approach compared to other types of microbial approaches, such as 16S rRNA gene sequencing. There is clearly the ability to obtain information about the taxonomic and functional activity of the sample and evaluate specific phylogenetic contributions within the data (Peters et al., 2019), substantially reducing the work involved in addition to imprinting greater sensitivity and relative accuracy.

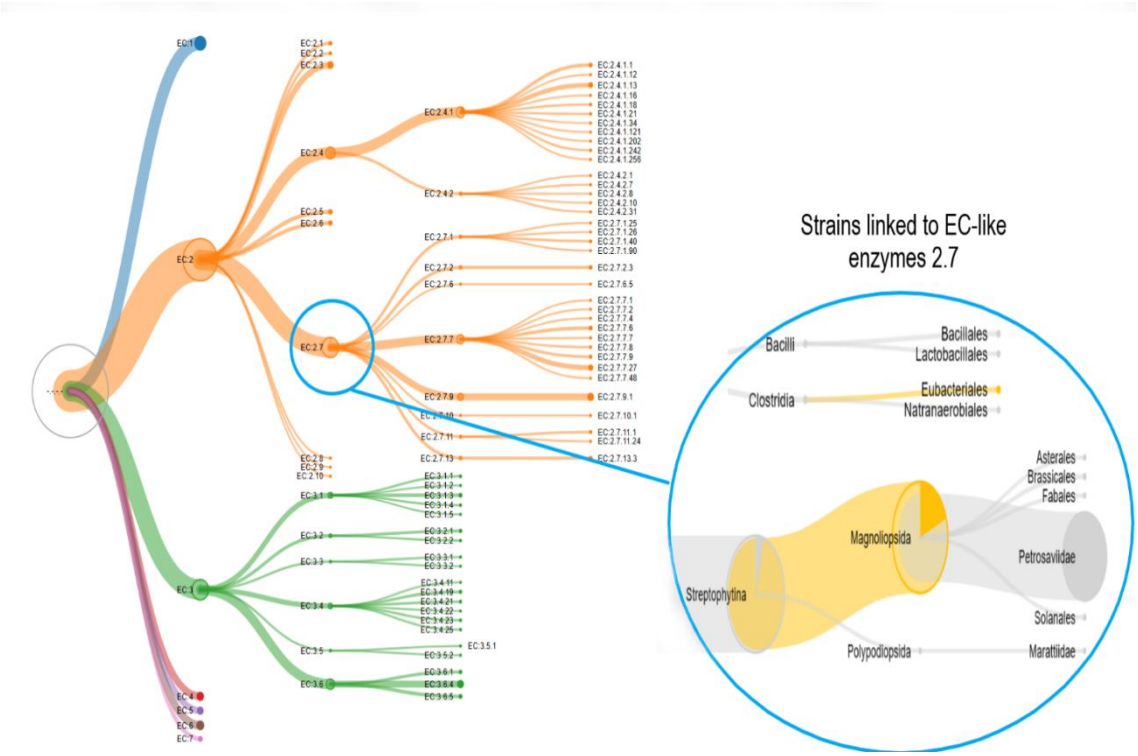

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Figure 4. Illustrative diagram of enzyme expression during and interconnection with the respective individuals (plants and microorganisms) at the beginning (24h)

The less in-depth analysis of enzymatic activity confirmed what was reported in a previous study (Bahule et al., 2023) about the possibility of enzymes acting at the substrate level, where they can disturb the microbial fermentation process and the transformation of the product. This scenario also affects the fermentation parameters since the obtention of organic acids, which guarantee the functional effect of the fermented product, and the necessary pH reduction, important for the conservation of the product, are also compromised.

Some strategies to better apply as eventual primary solutions to start a process of optimization are the heat pre-treatment of the corn; this will reduce the action of the substrate enzymes, eliminate a possible harmful microbiota, and allow the safe use and better control of the fermentation using a starter (Bahule et al., 2023).

In this approach, to perform an optimization process, a maize fermentation was performed using the Pediococcus acidilati bacterial strain, and a thermal pre-treatment of maize (121°C) was also performed to suppress the enzymes and obtain a basal metabolic expression of extracellular proteins from that starter bacteria. The treatments performed were: pre-treated and inoculated corn (T+I), untreated and inoculated corn (nT+I), and control (nT+nI). A multifactor ANOVA analysis was performed, and the factors (treatment, pH, Ac. L., and fermentation time) were compared with each other. The summary of these fermentation parameters is presented in the graph below (Table 1).

Table 1. Anova multiple factors: fermentation time (Time), treatments, pH (hydrogen potential), L.ac (lactic acid), and fermentation temperature (Temp)

All factor

	Mean	std. Desv
Treatment	102.0000	0.828079
Time(h)	102.5000	1.133893
pH	0.0000	1.000000
L.ac	0.0000	1.000000
temp(°C)	0.0000	1.000000

	Treatment	Time(h)	pH	L.ac	Temp(°C)
Treatment	1.0000	0.0000	0.1199	-0.4464	-0.3593
Time(h)	0.0000	1.0000	-0.5326	0.4830	-0.3837
pH	0.1199	-0.5326	1.0000	-0.7987	-0.0465
L.ac	-0.4464	0.4830	-0.7987	1.0000	0.2766
temp(°C)	-0.3593	-0.3837	-0.0465	0.2766	1.0000

Source: elaborated by the author

The result shows that the data had a normal distribution and that the means differed within each other. There is a strong correlation verified between pH and lactic acid (r = -0,7987), when the decrease of the pH value results in the increase of lactic acid. For better analyses of the factors, an ANOVA with repeated measurements was performed to see the behavior of pH and lactic acid within the three treatments, and the results are shown in the following graphics.

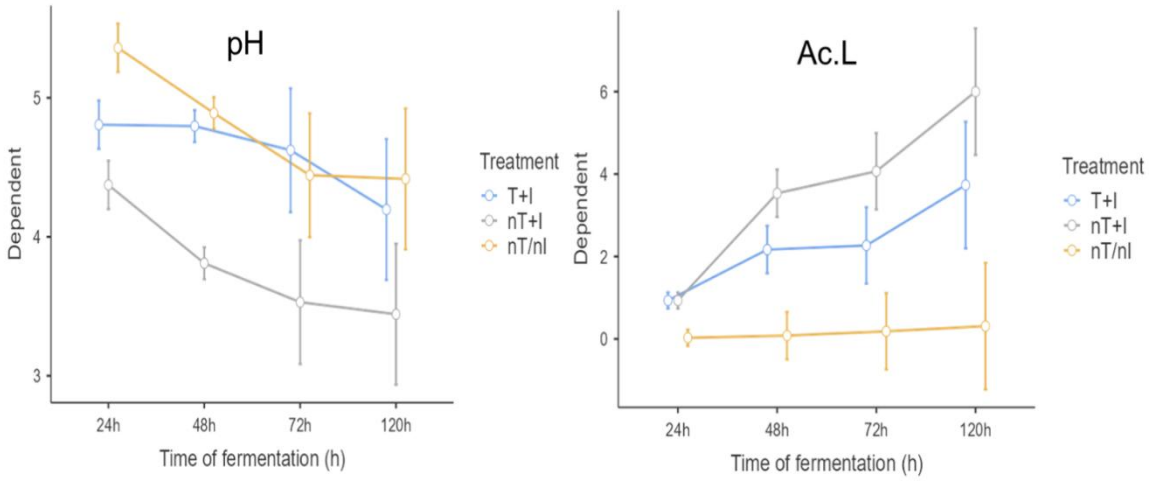

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Figura 5. Graphic of anova repeated measurement: behavior of pH (hydrogen potential) and lactic acid (L.ac) within the three treatment, treated and inoculated maize (T+I); non treated and inoculated maize (nT+I); non/treated and non/inoculated maize (nT+nI);

The pH and lactic acid are important parameters for the fermentation process since there is a need to have a sufficiently low pH to control deteriorating mesophiles, and these values are achieved in the range of 4.5 to 3.5, with 4.5 being the minimum required to ensure safety and 3.5 being the maximum accepted so as not to impair the sensory aspects and palatability of the product. These values strictly depend on the presence of organic acids (measured based on lactic acid); this measure has an inverse proportionality relationship with the pH since the increase in lactic acid leads to an increase in acidity and therefore a lower pH.

In the graph, it is possible to notice the slight reduction in pH and the almost insignificant concentration of lactic acid during the spontaneous fermentation time (nT+nI), where at the end of 120 h the pH was fixed close to the minimum values. The other treatments, such as T+I and nT+I, that received inoculation with Pediococcus acidilati had a rapid and satisfactory increase in lactic acid and therefore a decrease in pH in the first 24 hours of fermentation. This shows that the application of the starter culture is indeed an interesting strategy, and Pediococcus acidilati responds to the proposed objective.

After studying the advantages of using the starter strain in conjunction with maize pretreatment, there was a need to decide which treatment to select (nT+I or T+I) to use in future research on kinetic optimization.

Based on the most correlated fermentation parameters (pH and L. ac), a separate analysis was carried out, and the results are shown below (Table 2). Both treatments (nT+I and T+I) had pH correlated with L.ac. at very significant levels: -0.867 for nT+I and -0.936 for T+I, with the correlation graph showing a linear behavior.

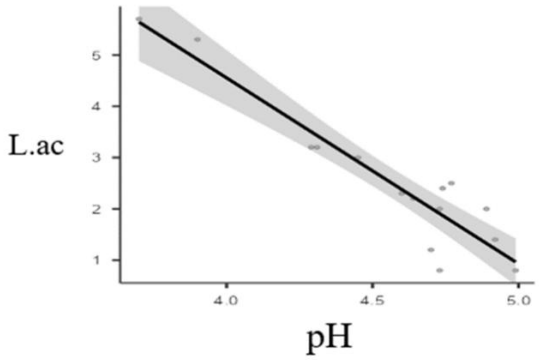
Table 2. Correlation analisys between maize fermentation parameters (pH, L.ac and Temp) in A non/pre-treated (nT+I) and B pre-treated(T+I) maize inoculated with *pediococcus acidilat* strain

Correlation Matrix (**nT+I**)

		pH	L.ac	Temp
pH	Pearson's r	—		
	p-value	—		
L.ac	Pearson's r	-0.867	—	
	p-value	< .001	—	
Temp	Pearson's r	-0.222	0.034	—
	p-value	0.427	0.905	—

Correlation Matrix **(T+I)**

		pH	L.ac	Temp
pH	Pearson's r	—		
	p-value	—		
L.ac	Pearson's r	-0.936	—	
	p-value	< .001	—	
Temp	Pearson's r	0.136	-0.090	—
	p-value	0.629	0.749	—



Temp: Temperature; pH: Hydrogenion potential; ; L.ac: Lactic acid
Source: elaborated by the author

In order to choose between the two treatments, other events must be considered in the evaluation, namely:

First: the non-heat treatment of corn: according to the evaluated parameters, the result obtained in nT+I was efficient; however, the inoculation of corn not previously treated is still not safe. Therefore, it assumes that there are synergies between microorganisms

and that harmonious growth between antagonistic strains is possible, and this has already been demonstrated in a study with metaproteomic analysis. Due to this, while the profile of these non-inoculated communities is not known, there will be limitations in the control and safety of the process.

Relative correlation value: analyzing the pH and L.ac correlation of the two treatments, it is clear that other factors affected the process. Where the R value of nT+I (-0.867) was relatively lower than T+I (-0.936), a choice between them would be the option for the highest value closest to 100%.

Probable endogenous corn enzymes: an inoculation made in an initially degraded substrate would be hypothetically better, as was the behavior of nT+I, but this is not the case. Not knowing the profile of the microbiota and the enzymes that will be involved in the fermentation, we assume that the expected result will also be unknown, even with a predicted trend.

6.4 Conclusion

The starter strain, Pediococcus acidilati, has been tested on several substrates but has never been tested on maize before. Its action on both pre-treated and non-treated corn was considered optimal, as it managed to reduce pH values to safe levels in the first 24 hours of fermentation. This shows that it is possible to obtain fermented corn for paste formulation in less time and with greater safety.

Considering all the assumptions, it should be noted that the pre-treatment of corn and its subsequent inoculation with an initiator strain is an approach that best presents itself in terms of advantages and results obtained. Metaproteomic once again showed the ideal ways, free from culture and a lot of work, but efficient and precise ways of optimizing maize fermentation.

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Conclusão geral

A metaproteômica como técnica livre de cultivo mostrou uma capacidade de traduzir os eventos ocorridos durante a fermentação e permitiu interpretar com maior precisão e rapidez, se comparado ao métodos convencionais de cultivo.

No geral, nossos resultados trouxeram uma contribuição para a compreensão de que processos fermentativos espontâneos carregam consigo uma grande complexidade e que a aplicação de métodos para obtenção de maior segunrança deve ser indispensável. Técnicas como a metaproteômica são úteis para o desenvolvimento de formulações melhoradas de ingredientes tradicionais e podem ser uteis na redução do tempo para analise e melhoramento de produtos tradicionais tal como os de milho fermentado. Embora a contribuição da metaproteomica seja evidente, os dados obtidos devem ser interpretados com cautela, considerando uma percentagem relativamente baixa (1 a 15%) de peptidios identificados e interligados a respectiva fonte. Além disso, mais estudos precisam ser realizados para entender melhor os mecanismos enzimaticos detalhados e os substratos alvos durante a fermentação.

Em estudos futuros, será importante associar a metaproteômica com outras ômicas como a metagenômica e a metabolômica, e fazer se uma comparação dos resultados destas ômicas para complementar as possíveis lacunas não explicadas com dados de metaproteômica.

O estudo comprovou o perigo referenciado em outras pesquisas que trabalharam com produtos de milho fermentado e como também mostrou que, alguns parâmetros de fermentação são essenciais para manutenção das propriedades e só podem ser conseguidos com a aplicação de iniciadores e pre-tratamento do substrato.

Os achados atuais suportam positivamente o uso de *Pediococcus acidilati* como potencial iniciador a ser utilizados como parte de estratégias do melhoramento da fermentação do milho. Por outro lado, *Pediococcus acidilati* mostrou-se uma cepa superior para fermentação de milho para produção de pasta alimentícia, com parametros bem estáveis e fermentação lática mais rápida.

Testes futuros podem explorar outras cepas LAB e encurtar o tempo de fermentação para menos de cinco dias.