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METABARCODING ANALYSIS AND OPTIMIZATION OF THE WATER KEFIR GRAINS FERMENTATION FOR THE DEVELOPMENT OF A STANDARD BEVERAGE

Presentation of the thesis for the awarding of the academic degree
DOCTOR IN BIOTECHNOLOGY

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Dr. Michael Seeger Pfeiffer

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To God and to my mother, Blanca Lucila Puerta, for giving me life and for showing me the light with which to walk.

ABSTRACT

Water Kefir Grains (WKG) are a microbial consortium consisting of lactic acid bacteria (LAB), acetic acid bacteria (AAB*), bifidobacteria* (BIF) and yeasts (LEV) embedded in a matrix of exopolysaccharides that convert sucrose into alcohols, organic acids and esters. WKG is considered a food with probiotic potential due to the microorganisms it contains. In Latin America, water kefir beverages are traditionally prepared in non-centrifuged sugar solutions (NCS), and some emerging companies have attempted to industrialize this process, facing difficulties in product standardization, which shows the need to develop methodologies that help stabilize this bioprocess. The qualities of WKG ferments can be influenced by higher order properties, factors that can be evaluated by response surface methodology (RSM) designs. RSM combined with metabarcoding are powerful tools to determine the optimal conditions for standardization of this biological process. Considering the above, the following hypothesis is proposed: "The optimization study in which different temperature and agitation conditions are evaluated, using RSM together with metabarcoding analysis, on the effect of physicochemical and microbiological composition on the fermentation of NCS with WKG, allows establishing the optimal operating conditions to obtain a fermented beverage with a standard sensory quality and with a known microbiological composition indicating a probiotic functional potential. As a methodology, first a selection of WKGs was made by studying the physicochemical properties between grains from Chile and Colombia; then the preliminary microbial composition of the selected grains was observed by metabarcoding. Subsequently, Central Composite Design (CCD) was used to evaluate the factors of agitation (0-160 rpm) and temperature (23-40 °C) in order to standardize the process by RSM, using metabarcoding, HPLC, and gravimetry. A substrate change study was also conducted to evaluate grain growth, LAB, BIF, and LEV counts, and microbial identification by MALDI-TOF and 16S rDNA gene sequencing. Finally, a marketing project for the fermented beverage was proposed. The main results were as follows: The selected WKGs were WKGCOL3 and WKGCH2, out of 5 possible candidates evaluated with scores of 35% and 20%, respectively. This selection was made based on better metabolic response and higher biomass yield. The preliminary metabarcoding analysis showed that 9 bacterial species (*L. ghanensis, L. casei/paracasei, L. harbinensis/perolens, L. hilgardii, L. diolivorans, L. parabuchneri, L. satsumensis, O. alcoholitolerans,* and *O. oeni*) and 6 species of yeasts (*D. bruxellensis, S. cerevisiae, Z. florentina, S. ludwigii, S. pombe*, and *C. californica*) were found in varying proportions for both selected grains. In the same preliminary study, it was found that varying the substrate and temperature caused significant changes in the relative abundance of yeast and bacterial species when evaluating the different SDs. In the optimization study, the main metabolites and sugars were generally found to vary as follows: Lactic acid ranged from 1.2 to 11 g/L, acetic acid from 0.2 to 1 g/L, and ethanol from 7 to 26 g/L. All these metabolites showed their maximum levels at the average temperatures evaluated (30 and 37 °C) and their minimum levels at 20 °C. For sugars, glucose ranged from 2 to 13 g/L and sucrose from 6 to 62 g/L. Both sugars showed their maximum consumption at 37°C and their minimum consumption at 20°C. Fructose was found to be consumed between 4 and 23 g/L, with a minimum consumption at 30°C and a maximum consumption at 40°C. Biomass of WKGs generally ranged from 92 to 150 g/L. Maximum growth occurred between 23 and 30 °C and minimum growth between 37 and 40 °C. Response variables generally showed good fits to quadratic and/or linear models with p-values <0.001 and correlation coefficients R^2 greater than 0.5. The metabarcoding optimization analysis found the same microbial species as the preliminary analysis plus the following microbial species: O. kitahare, and L. perolens. The optimal general operating point found was 54 rpm and 37°C with a global desirability (GD) of 0.63. The substrate change analysis to evaluate the biomass increase showed that NCS is a substrate that allows the greatest increase compared to refined sugar. Microbial analysis by MALDI-TOF additionally identified *L. nagelli, Z. mobilis, and A. fabarum, A. ghanensis, A. lovaniensis, A. orientalis*, and *G. oxydans*; and 16s RNA analysis identified *L. hilgardii, Liquorilactobacillus nagelii*, and *Bifidobacterium aquikefiri*. The economic projection and market study showed that the business idea is viable and can represent a significant intervention in the functional beverages sector, with a positive net present value (NPV) of 49 million (CLP), demonstrating feasibility and return on investment within 4 years. It is concluded that it is possible to standardize water kefir fermentation using RSM under the aforementioned operating conditions. Furthermore, it was observed that WKG, regardless of its origin, behaves similarly under most of the conditions studied, indicating that these grains may have the same origin. It can also be said that the polynomial equations found by RSM can be used for prediction purposes in processes with WKG of different origins. The microorganisms identified are mostly *Lactobacillus* species, which may have probiotic potential.

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LAURA J. MUÑOZ PUERTA

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LIST OF ABBREVIATIONS AND UNITS

AA	Acetic Acid
AAB	Acetic Acid Bacteria
Adeq	Adequate
ANOVA	Analysis of Variance
BIF	Bifidobacteria
CCD	Central Composite Design
Ca	Calcium
CAGR	Compound Annual Growth Rate
CFU	Colony Forming Unit
CLP	Chilean Peso
CO_2	Carbon dioxide
Cu	Copper
CV	Coefficient of Variation
DNA	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharides
EtOH	Ethanol
EXP	Experiment
Fe	Iron
FG	Final Grain
FRUC	Fructose
FTIR	Fourier Transform Infrared Spectrometry
g	Grams
GCY	Glycerol casamino yeast extract culture medium
GI	Gastrointestinal
GD	Global Desirability
GLUC	Glucose
GRAS	Generally Recognized as Safe
h	Hours
K	Potassium
kDa	Kilodaltons
K_2HPO_4:	Dipotassium phosphate
L:	Liter
LA	Lactic Acid
LAB	Lactic Acid Bacteria
MALDI-TOF	Matrix Assisted Laser Desorption/ionization Time of Flight mass spectrometry
mg	Milligrams
Mg	Magnesium
min	Minute
mm	Millimeters
Mm	Millimolar
mL	Milliliters
MRS	de Man, Rogosa and Sharpe culture medium
mTY	Modified Tryptone - Yeast extract culture medium
N_2	Nitrogen
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NCS	Non-Centrifugal Sugar
NPV	Net Present Value
nr	Non-reported
PCR	Polymerase chain reaction
pH	Potential of hydrogen
pmol	Picomole
PLA	Poly lactic acid
R^2	Correlation coefficient
RSM	Response Surface Methodology
rpm	Rotations per minute
SUCR	Sucrose
T	Temperature
TE	Tris and EDTA Buffer
WKG	Water Kefir Grains
WKGALE	Germany Water Kefir Grains
WKGOL3	Colombian Water Kefir Grains, number 3
WKGCH2	Chilean Water Kefir Grains, number 2
v	Volume
w	Weight
YPG	Yeast Extract – Peptone Glycerol culture medium
Zn	Zinc
°C	Degree Celsius
µg	Microgram
µL	Microliter

1. INTRODUCTION

1.1. HISTORY AND PROSPECTS OF NON-CENTRIFUGAL SUGAR (NCS) FERMENTATION WITH WATER KEFIR GRAINS

Fermented foods and beverages were among the first processed foods consumed by humans, i.e., those whose production involves the action of microorganisms or enzymes that cause desirable biochemical changes and significant modification of the food. Their production and consumption date back thousands of years, with early evidence of alcoholic fermentation of barley to beer and grapes to wine (Campbell-Platt 1994), records of alcoholic fermentation of beer and wine date back some 5,000 years (Borgstrom 1968; Tamang and Kailasapathy 2010), at a time when drinking water supplies could not be guaranteed, so the availability of fermented beverage, fermentation is then one of the oldest known biotechnological processes, this "traditional biotechnology" has developed through the knowledge of natural processes and environmental conditions that favor the selection of microorganisms for use as starter cultures or for improvement through genetic engineering.

Fermentation processes are currently used to process a wide range of foods and by-products. The improved shelf life, food safety, and organoleptic properties of fermented foods have increased their value, and these foods are in the spotlight for their nutritional value, functionality, and improved health. Historically, fermented foods were obtained through spontaneous fermentation processes, but many of these traditional processes are still poorly understood or uncontrolled (Munanga *et al.*, 2016). Today, most known fermented beverages are produced industrially using starter cultures, resulting in faster processes and more consistent and safer end products (Laureys and De Vuyst 2017).

Natural fermentation processes are often complex and not yet well understood, but the tools and methods available to study microbial ecology are still evolving and are showing promising results to understand in depth the functioning of these microbial ecosystems and, therefore, to optimize naturally occurring fermentations with a deep understanding of the process (Laureys and De Vuyst 2017). One of the best-known naturally fermented products is kefir, a handmade potential probiotic food that is widely consumed around the world. This beverage can be found in three varieties: milk kefir natural starter culture, which contains a diverse range of indigenous lactic acid bacteria, acetic acid bacteria and yeasts in a polysaccharide matrix of semi-hard grains called kefiran, which originated in the Caucasus (Abadl *et al.*, 2022; Satir and Guzel-Seydim 2016); tea kefir, known as "kombucha", transforms tea solutions

and contains the same microbial groups but is embedded in a cellulose polymer (Chakravorty *et al.*, 2016). The third variety is water kefir grains (WKG), which has a microbial community composed of lactic acid bacteria, acetic acid bacteria, *Bifidobacteria* and yeasts embedded in a macroscopic exopolysaccharide (EPS) matrix of α-1,3-linked glucose (Figure 1.2) (Fels *et al.,* 2018) and the α-1,6-glucose polymer dextran (Laureys and De Vuyst 2017b; Gulitz *et al.,* 2011). This matrix consists of macroscopic grains, a gelatinous structure with a diameter of 5 to 20 mm and an irregular, cauliflower-like shape (Waldherr *et al.,* 2010). The structure is transparent and brittle, insoluble in water, and sinks to the bottom of the fermentation liquor (Laureys and De Vuyst 2017b; Waldherr *et al.,* 2010; Anna Gulitz *et al.,* 2011). WKG transforms aqueous sugar solutions into various organic compounds such as alcohols, organic acids and esters. Traditionally, water kefir beverages are made in Europe and Asia by fermenting refined sugar solution substrates with the addition of various fresh or dried fruits at room temperature for one to four days under anaerobic or aerobic conditions. The result is a cloudy, blond-colored, carbonated, acidic, and slightly alcoholic, low-sugar beverage containing viable cells of lactic acid bacteria and yeasts (Anna Gulitz *et al.,* 2011; Waldherr *et al.,* 2010).

In some South American countries, such as Chile and Colombia, water kefir beverages are prepared in an artisanal way by depositing this type of grain in a Non-Centrifugal Sugar (NCS) cane or beet solution (Caro Vélez and León Peláez 2014), obtaining an acidic, slightly alcoholic, carbonated, light brown beverage. Water kefir beverages are sensory pleasing and complex; they may contain phenolic compounds, flavonoids, anthocyanins, and antioxidants in addition to organic acids, ethanol, and CO_2 (Estabeyoglu *et al.,* 2023). For example, the sensory profile in WKG fermentation of different substrates is to a greater extent due to the presence of the microorganisms involved. In the case of CO_2 production, it is well known that its metabolic process starts with the conversion of fructose to glucose by the enzyme glucose isomerize. *Bacillus* spp., *Acetobacter* spp., and *Lactobacillus* spp. are known to produce this enzyme, species found in water kefir grains (Waldherr *et al.,* 2010). On the other hand, flavor development of water kefir fermenters also occurs when glucose is used by LAB to produce lactic acid and when yeasts use glucose to produce ethanol and carbon dioxide gas (Surja *et al.,* 2019). Therefore, it is relevant to investigate how the abundance of microbial groups and species present in the WKG intervene in the development of flavor to determine the conditions for obtaining fermented products with specific characteristics that correspond to the microbiological composition and proportion of these beverages.

According to the Food and Agriculture Organization of the United Nations FAO, NCS is rich in nutrients such as sucrose, minerals, amino acids, polyphenols, and vitamins (Colina *et al.,* 2012; Jaffé 2015; Weerawatanakorn *et al.,* 2016). This NCS is not expensive (low cost), and its fermentation capacity is

currently not fully understood. Therefore, it represents a unique opportunity for the development of new functional foods that add value to this sweetener. There is interest in replacing refined sugar with sweeteners of "natural" and "organic" origin, as these are becoming increasingly popular and have significant market shares in many countries. In contrast, products containing refined sugar are negatively perceived as being associated with obesity, metabolic syndrome and fatty liver disease (Stanhope 2016), opening an opportunity for diversification and production of products containing unrefined sugars (Jaffé 2015; Jaffe 2012).

One of the main issues in the emerging industry of kefir-derived beverages is the composition, organoleptic qualities and standardization of their processes. Within this topic, the low grain growth (Marsh *et al.,* 2013) and the variability of fermentation conditions in specific communities emerge as problems to be solved (Laureys and De Vuyst 2017; Dertli and Çon 2017). The organoleptic profile of these fermented foods could be influenced by factors such as temperature, agitation, inoculum, and substrate concentration, among others (Leroi and Courcoux 1996; Gao *et al.,* 2012). Therefore, kefir fermentation parameters can be adjusted to achieve a desired standardization (Mehran Ghasemlou and Khodaiyan 2012). Mathematical modelling of these processes has several advantages: control through silico simulations, reduction of experimental costs, optimization, among others (Garre *et al.,* 2019). The kinetic behavior of milk kefir fermentations has been extensively studied to predict biomass increase under linear and non-linear models (TramŠek and GorŠek 2008), but no yet the water kefir fermentation kinetic behavior. Another method used in experimental designs for kefir fermentation is the use of response surface methodology (RSM) (M. Ghasemlou, Khodaiyan, and Gharibzahedi 2012; Gao *et al.,* 2012), this tool has been developed to correlate the responses of the system with the dependent or manipulable variables and naturally emerge in the optimization, that is, the search for an operating point in these fitted surfaces (models) that satisfies constraints such as cost minimization and the desired concentration of molecules involved in the sensory experience of the consumer (Myers, Khuri, and Carter 1989).

Understanding the need to standardize the fermentation of WKG to obtain non-dairy foods with probiotic potential made with NCS, the evaluation of exogenous factors such as temperature, agitation and available oxygen was proposed and through mathematical models derived from RSM, the best conditions were predicted that allowed the standardization of the fermentation of NCS beverages with WKG. The exogenous factors were evaluated in such a way that the grain grows properly, and the formation products impart the best sensory fermentation qualities. The external variables that can be used to control a fermentation process, such as the water kefir consortium, are factors that influence important properties of the microbial community, such as stability. Higher-order properties, according to the theory of

microbial community ecology, arise from the interaction of community members and are not predictable by examining each member individually; these properties can be aspects such as stability and are mainly a consequence of the diversity of the microbial community (Loreau and de Mazancourt 2013). Some interactions between members generate internal or endogenous dynamics in the microbiological composition of the community that are perturbed by factors external to the system, which is the basis of stability. Optimization of community stability and process standardization can be achieved by elucidating the factors that control these properties or operating conditions. If the physicochemical environment is constant, the composition of microbial communities would be in continuous flux due to controlled endogenous dynamics, which is reflected in the relative abundance of members; therefore, microbial communities can be considered complex adaptive systems in which exogenous factors modulate endogenous dynamics (Konopka, Lindemann, and Fredrickson 2015). Therefore, standard fermentation can be achieved if the fermentation can be controlled by exogenous factors such as agitation or temperature.

Metabarcoding has been recognized as a suitable technique to study the compositional dynamics of a microbial community; in studies with water kefir from native German grains, pyro-sequencing was performed, providing information on the relative abundance and diversity of microbial species present in the grain and allowing to understand the uncontrolled natural dynamics of fermentation (Stadie *et al.*, 2013; A. Gulitz *et al.*, 2013; Laureys and De Vuyst 2017). On the other hand, assessing microbial consortia and using mathematical models can provide a better predictive understanding of consortium dynamics (Henson and Hanly 2014). The perspective of using predictive models is well complemented by genomics, which expands the knowledge of omics, kinetic, and physiological data and helps to better predict and describe the behavior of microbial communities under given operating conditions, thus enabling the determination of the most appropriate exogenous parameters that confer process stability (Konopka, Lindemann, and Fredrickson 2015). Advances in the ability to control the stability of microbial ecosystems include approaches from methodologies that control external conditions to the system, which have proven successful through environmental control and selective pressure (Minty *et al.*, 2013).

Understanding the need to standardize fermentations with WKG, it is proposed to use techniques such as metabarcoding to comprehend the endogenous microbial composition, and the use of empirical mathematical models derived from RSM to help predict the best operating conditions to achieve the desired compositional and microbiological profile. The metabarcoding study can also identify novel microorganisms that have technological utility in food processes and provide information on the

relationship between microbial composition and environmental control factors (such as temperature, agitation, oxygen intake to the system, among other factors), a relationship that speaks to the relative abundance of microbial species in the microbial community according to the modifications of external factors.

In Europe, Chile and Colombia, water kefir fermentation with NCS is a non-industrialized process and has presented difficulties in grain growth and product standardization, showing the need to develop methodologies to control this bioprocess. The physicochemical and microbiological characteristics of this food are influenced by the concentration and composition of the inoculum and substrate, agitation, temperature and the amount of oxygen. These factors can be modified, and thus RSM designs find the operating points that satisfy the expected results. RSM coupled with techniques such as metabarcoding can become powerful tools to determine conditions for the optimization of fermentation biological processes. The main objective is to determine the optimal operating conditions for fermentation with WKGs and the microbiological composition of this consortium, using NCS as substrate, thus obtaining valuable information that can be used for the standardization of this type of beverages.

1.2. KEFIR LIKE NATURAL FERMENTED BEVERAGE AND ORIGINS

The origin of water kefir is unclear. It has been documented that Balfour first named water kefir as "ginger beer plant" in 1887. It may have been introduced to Britain around 1855 by soldiers returning from the Crimea and resembled the grains used to make "kephir," a milk beverage described by Kern in 1881 (Kleber 1921; Ward Marshall 1892). In addition, water kefir is reported to have originated in the Caucasus region (Pidoux, Brillouet, and Quemener 1988). In parallel, it has been reported that the grains were found in the fruit of the cactus opunti or "tuna" in Mexico, calling them "tibi grains" (Pidoux 1989). This microbial community has been called "sugar kefir", "California bees", "African bees", "Japanese beer grains" (Horisberger 1969; Pidoux 1989), among others, to distinguish water kefir from milk kefir. The various speculations on the origin of "water kefir" indicate that it cannot be attributed to a specific source of this microbiota (Laureys and De Vuyst 2017a). The grains can be reused for successive fermentations by "back-slopping," i.e., using grains from the previous fermentation and placing them on a new substrate; this could be practiced ad infinitum (Pidoux, Brillouet, and Quemener 1988). Traditionally, these grains are passed on or shared from one generation to the next; water kefir consumption is traditionally high in South America, Eastern Europe, and Russia.

According to Fiorda (2016), kefir grains were passed down from generation to generation among tribes in the Caucasus (red area in Figure 1.1) and were considered a source of family wealth. From there, kefir grains were distributed to countries on the European, African, and Asian continents. As the habit of drinking kefir spread throughout Europe, kefir grains reached the "New World", and its use spread throughout the American continent. Today, kefir is made in homes around the world by culturing fresh or pasteurized substrates with kefir grains. The green area in Figure 1.1 shows the countries with the highest consumption of water kefir, including the United States, Mexico, Canada, Thailand, Malaysia, Japan, Greece, Turkey, the United Kingdom, the Netherlands, Norway, Sweden, Spain, Chile, and Peru.

When kefir grains are used to ferment fruit juice, molasses, or a sugary solution, it is called sugar kefir or water kefir (Magalhães *et al.*, 2010). WKG have been adapted to many names because they have been around for so long and are shared by so many cultures around the world. Some of the names are like milk kefir because of the lack of distinction between the two through history (just as both are called "kefir" but only distinguished by saying milk or sugary). The history of water kefir is not well known, but according to Fiorda 2016 (Fiorda *et al.*, 2016), although it's structurally like milk kefir, the origin, distribution, and consumption drew an individual path.

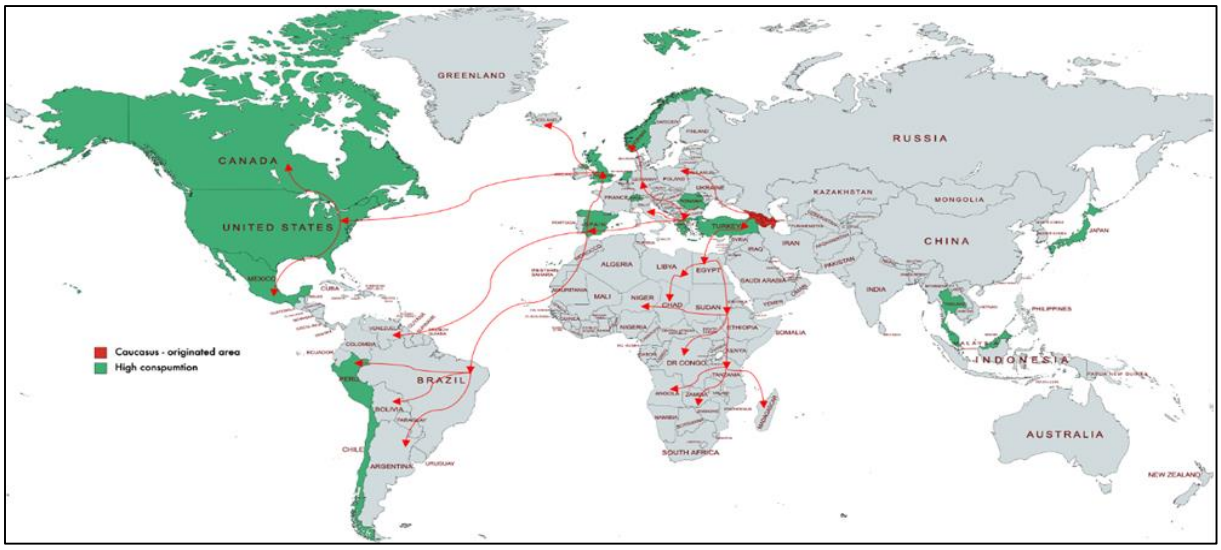

Figure 1.1. *Origin, distribution and consumption of water kefir. *Red area - area of origin (Caucasus); *Green area - high consumption. (Fiorda, 2016). This figure explains how kefir grains have spread around the world from their region of origin in the Caucasus Mountains.*

Kefir as a natural or industrial fermentation technique has been developed in different cultures around the world, starting from different raw materials and techniques, so fermented foods derived from these grains vary from continent to continent (Hutkins 2008; Tamang *et al.*, 2016). Spontaneous processes depend on several environmental factors, indigenous microorganisms, and the nature of the products to

be fermented (Leroy and De Vuyst 2004). These fermentation products can result in healthy, sensorially pleasing foods, but they can also become unsafe for human consumption (Nout, 1994), so it is important to comprehensively study the microbial components of foods such as kefir to provide a safe product.

Concepts such as sensory quality, functionality and food safety are currently of great importance because they are relevant to understanding the wide variety of foods that can be of natural or industrial origin and that are inherent to each culture. According to the FAO, food security is defined as: "At the level of individuals, households and nations, it is achieved when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life". In accordance with the above, the policies of countries should promote both innovative and processed foods as well as give value to the nutritional quality of natural fermentations and what they can offer at the level of health using current technological advances to ensure the consumption of indigenous foods and that favor what food security regulates for all cultures in the world. This prelude to the concept of food security is relevant to the purpose of this thesis, since the microbial communities of water kefir have been analyzed to verify the convenience in the development of a standard beverage that provides access to traditional and culturally accepted food.

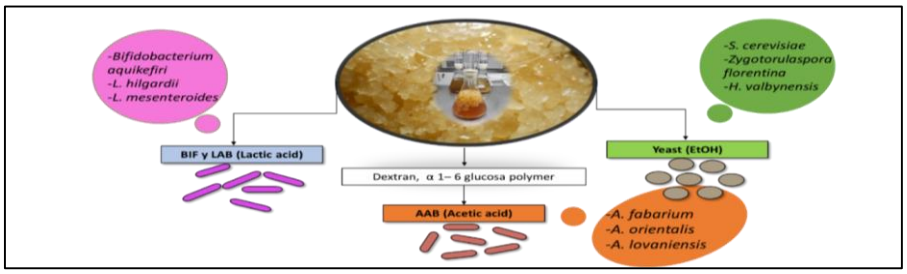

Figure 1.2. *Description illustrating the microbiological composition, main metabolites, and dextran of water kefir grains (WKG), a microbial community composed of Bifidobacteria (BIF), lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeasts embedded in an exopolysaccharide matrix.*

1.2.1. Water Kefir Fermentation

The Water Kefir Grains are used as inoculum for the next fermentation process (back-slopping). Fermentation is usually done in a container with a gas trap to promote anaerobiosis or in the presence of oxygen using a cloth to cover the container. (Reifl 1990). Figure 1.3 shows the fermentation models used in this study with WKG from different locations in Colombia and Chile. Volumes of 2 liters and 250 mL can be seen, equipped with an airlock promote anaerobiosis (this is to prevent the development of acetic acid bacteria and ensure the sensory quality of the beverage). It was noticed that the WKG not only remained at the bottom of the flask, but some also floated on the surface while others moved vertically

between the two ends of the flask. The floating grains were larger in size and showed greater activity with more obvious gas production.

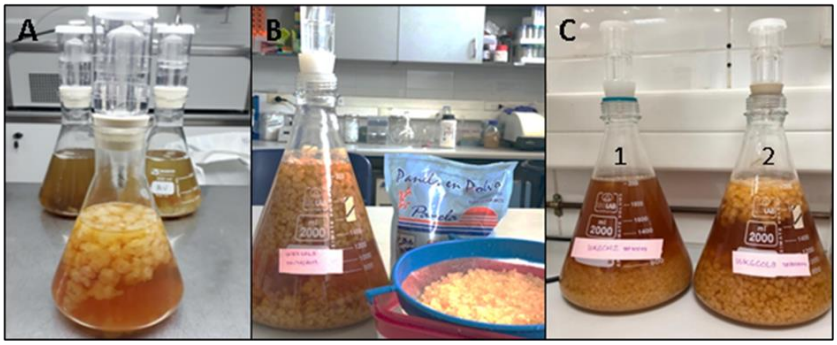

Figure 1.3. *Anaerobic fermentations of water kefir with unrefined sugar (NCS) at different operating volumes. (A), and (B) fermentations of Colombian kefir grains at scales of 250 mL and 2000 mL, respectively. (C) 1. Fermentation of Chilean kefir grains and 2. Fermentation of Colombian kefir grains at 2000 mL scale.*

1.3. FERMENTATION PROCESS IN LATIN AMERICA

In Latin America, water kefir ferments are elaborated using kefir grains with low-cost raw materials to obtain economical products (Caro Vélez and León Peláez 2014). One of these raw materials is the non-centrifugal sugar (NCS), the technical name used by the FAO, which is a solid, unrefined product obtained by evaporating sugarcane or sugar beet juice (FAO, 1994). It is traditionally consumed as a sweetener in most sugarcane growing regions of the world. It has a brownish color and is rich in nutrients. In NCS, sucrose is the major component, ranging from 76.55 to 89.48%, followed by reducing sugars (3.69 to 10.5%) and water (1.5 to 15.8%). The mineral content (ash) is relatively high (0.3 to 3.6%), protein content ranges from 0.37 to 1.7% and fat from 0 to 0.1% (Weerawatanakorn *et al.*, 2016), and minerals (K, Na, Ca, Mg, Fe, Cu, Zn) are on average 285 mg/100g, equivalent to 0.0003% (Weerawatanakorn *et al.*, 2016). The basic difference between NCS and refined sugar is the presence of reducing sugars and significant amounts of minerals and other minor components. The nutritional and functional differences depend mainly on these components. So far, there is no known diversity in the bio-transformations of unrefined sugar such as NCS, which represents an opportunity for the development of new functional foods that add value to this product (Jaffé 2015). The industrial subgroup producing panela represents the second most important agribusiness in Colombia, after the coffee industry, due to the generation of more than 855,365 jobs, panela is produced in the typical facilities called "Trapiches Paneleros". Colombia produces more than 1.2 million tons of panela, of which 1% tons were exported to different countries around the world (Minagricultura 2019).

1.3.1. Panela to produce kefir beverages

Panela is a sweetener obtained from sugar cane (*Saccharum officinarum L*), widely consumed in Latin America, and used to sweeten juices, tea, infusions, soft drinks, jams, cookies, etc. In Colombia it is a popular beverage known as "agua de panela" or "aguadulce" (a mixture of water and panela). Even though it is a substrate rich in fermentable sugars and micronutrients, so far, its use as a substrate for the elaboration of fermented foods with kefir grains has not been well documented. Only the use of fermented panela with water kefir grains has been reported to evaluate contains of antimicrobial organic acids with antifungal capacity, specifically against the in vitro growth of *Aspergillus ochraceus* (Caro Vélez and León Peláez 2014). Figure 1.4 shows the general process for the water kefir fermented beverages production.

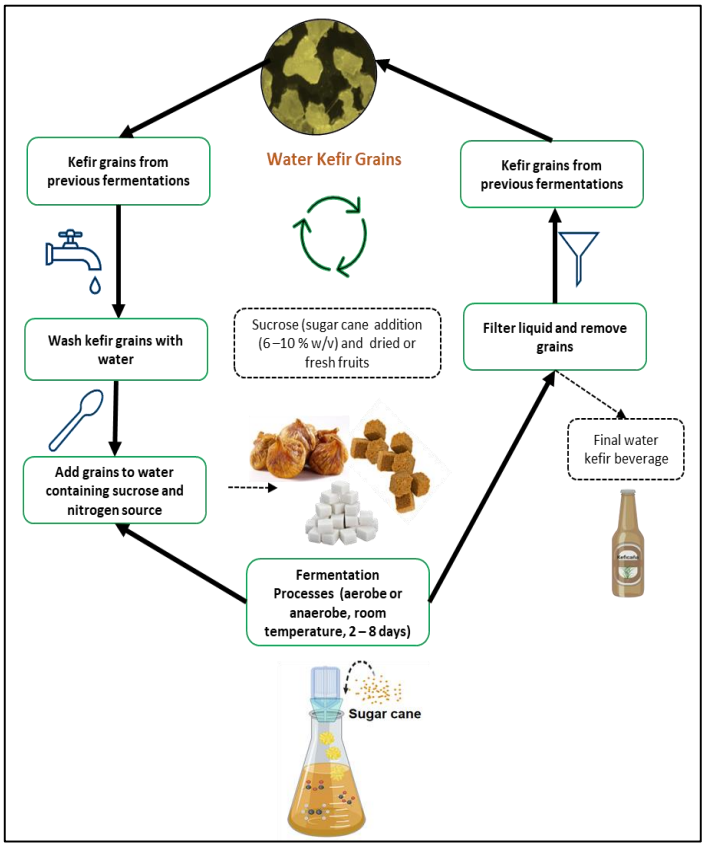

Figure 1.4. *Step-by-step description of the back-slopping process of fermentation with water kefir grains from sugary substrates. In the diagram, the process begins with the use of water kefir grains from previous fermentations, which are washed with preferably sterile drinking water. Then a mixture of sucrose (6-10%) is added along with a nitrogen source (dried or fresh fruit). This mixture is brought to the desired state (anaerobic/aerobic, at room temperature, for a period of 1 to 8 days), during which time the grains consume the sugars and produce carbon dioxide, alcohol and various organic acids. Then the kefir grain is separated from the already fermented substrate, and this fermented beverage is the final product that is consumed as a beverage with functional potential. The filtered grains serve as a starter culture for a new fermentation.*

1.4. KEFIR AS POTENTIAL PROBIOTIC

Probiotics were defined in 2001 by a United Nations Food and Agriculture Organization/World Health Organization expert consultation. In 2013, ISAPP convened an expert panel to review probiotics and literature. The result was a publication that restated the definition with minor grammatical changes: "Live microorganisms that, when administered in appropriate amounts, provide a health benefit to the host." This is the widely accepted scientific definition of probiotics worldwide. The history of the concept of probiotics dates to 1908, when the beneficial effects of some fermented foods on human health were described (Metchnikoff 2004), and numerous subsequent studies have confirmed this observation. The main criteria for choosing a probiotic are that these microorganisms have resistance to gastric acid and bile salts, adherence to intestinal surfaces, self-aggregation, co-aggregation, and antimicrobial activity against pathogens (Devi, Archer, and Halami 2015). The most used probiotic microorganisms belong to the genera *Lactobacillus, Bifidobacterium, Pediococcus, Streptococcus, Lactococcus*, and *Enterococcus*, and their most recognized health-promoting effects include relief of symptoms of inflammatory bowel disease and colitis, immunomodulation, protection against diarrhea, relief of lactose intolerance, allergy prevention/reduction (Soccol *et al.,* 2010) and cholesterol reduction (Kumar *et al.,* 2012). Many probiotic strains, especially those belonging to the genera *Bifidobacterium*, and *Lactobacillus,* have been used by the food industry because they are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) and because of the benefits attributed to them (Pray, Pillsbury, and Tomayko 2013; Granato *et al.,* 2010). Some strains of *Bifidobacterium* are used commercially due to their positive impact on human health and are prominent in functional foods. (Turroni, van Sinderen, and Ventura 2011).

While fermentation can extend shelf life, it can also enhance the flavor, aroma, and texture of finished products (Hutkins 2008). Fermented foods and beverages have a long history of beneficial effects on human health due to the microorganisms they contain or their metabolites (Metchnikoff 2004; Stanton *et al.,* 2005). A fermentation process can improve nutritional value, promote health through the production of vitamins and trace elements (Stanton *et al.,* 2005), bioactive peptides (Seppo *et al.,* 2003), antioxidants (Bernaert *et al.,* 2013), compounds with therapeutic or prophylactic properties (Parvez *et al.,* 2006) by the microorganisms involved in fermentation. Specifically, the capabilities of fermented foods such as water kefir have been linked to the presence of lactic acid bacteria strains in these microbial communities. Given these qualities, the current consumer is more open to the benefits of fermented foods, especially if the food has a traditional development and if they have qualities such as "lactose free", "suitable for vegans" among others (Lynch *et al.,* 2021). As a result, it is widely known that water

kefir is an excellent source of strains of microorganisms with potential health benefits. Although most of the reported kefir studies have focused on milk kefir and the different dairy substrates used (Prado *et al.*, 2015; Magalhães *et al.*, 2011; Kabak and Dobson 2011; Garofalo *et al.*, 2015; Bensmira, Nsabimana, and Jiang 2010), water kefir grains strains microorganisms have been studied for new food products with probiotic potential, as there is some background on the functional properties of this consortium. Potential probiotic effects (Zanirati *et al.*, 2015) and functional activities such as anti-hyperlipidemic, anti-hyperglycemic (Alsayadi *et al.*, 2014), immunomodulatory (Calatayud *et al.*, 2021), antimetastatic (Zamberi *et al.*, 2016), anti-inflammatory, and healing (Moreira *et al.*, 2008) have been attributed to microorganisms isolated from water kefir and the polymer containing them. However, little or nothing is known about the functional - probiotic effect that foods obtained from fermentation with this type of microbial consortium may have.

The microbiota associated with water kefir has mostly been studied from European grains (Pidoux 1989; Anna Gulitz *et al.*, 2011), and the microbial genera found in consortia from this continent have been determined. In water kefir, the genera *Lactobacillus, Acetobacter, Leuconostoc, Bifidobacterium, Saccharomyces, Pichia, Lanchancea, Kluyveromyces, Kazachstania, Hanseniaspora* and *Zygotorulaspora* have been identified, with about 27 different species of bacteria and 11 of yeasts (Fiorda *et al.*, 2017; A. Gulitz *et al.*, 2013; Stadie *et al.*, 2013b; Anna Gulitz *et al.*, 2011). Some of the species found in this consortium have been studied, either for their fundamental role in grain growth or as strains of microorganisms that may help to confer functional properties to this bacterial niche. *Lactobacillus hilgardii* has been identified as one of the most important for the grain growth or augmentation phase, since its abundance is related to the increase in biomass (David, Luc, and W. 2014; David Laureys *et al.*, 2017) and to the formation of the exopolysaccharide dextran, which serves as an immobilization support for all the microorganisms present (Pidoux 1989; Waldherr *et al.*, 2010). On the other hand, in even more recent studies, *Bifidobacterium* has been identified as a new genus in this community, attracting special attention for its potential probiotic and GRAS character (Ladero and Sánchez 2017); *Bifidobacterium psychraerophilum* (Gulitz *et al.*, 2013), *Bifidobacterium crudilactis*, and a new microorganism named *Bifidobacterium aquikefiri* sp. nov., have been identified and isolated from water kefir grains.

1.4.1. Lactic-Acid Bacteria as Probiotic

LAB are gram-positive, catalase-negative, non-spore forming, usually non-motile, non-respiring rods *or coccobacilli*, non-acid-fast, and cytochrome-free. They grow under anaerobic

conditions, but can grow under microaerophilic and aerobic conditions, with optimum growth at less acidic conditions (pH 5.5 - 6.0). They are strictly fermentative, with lactic acid as the main product, and have two different metabolic pathways (hexose fermentation). In the homofermentative pathway, lactic acid is the main product, whereas in the heterofermentative pathway, lactic acid, ethanol, acetone and CO_2 are the final products (König and Fröhlich 2009). Lactic acid bacteria (LAB) as indigenous, are distributed in nature, as natural contaminants in raw milk, yogurt, cheese, etc., and sometimes with the coexistence of yeasts, molds and some other pathogenic or non-pathogenic microorganisms, as is the case of WKG, LAB constitute a ubiquitous bacterial group that is widespread in nature in niches of the gastrointestinal and urogenital tract of humans and animals, and soil and water (Liu *et al.,* 2014).

Most dairy industries use LAB as starter cultures to produce fermented products, including dairy products such as yogurt, cheese, meat products, bakery products, wine and vegetables, contributing to the development of the product's taste, flavor, aroma, viscosity and texture (Khalil and Anwar 2016), as starter cultures, as probiotics, and in the production of interesting compounds (i.e., nutraceuticals) due to their versatile metabolism (Ruiz *et al.,* 2017; Emerenini *et al.,* 2013; Naeem *et al.,* 2012). Lactic acid bacteria possess enzymes that allow them to metabolize different compounds, which can increase or decrease the bioavailability of bioactive compounds, antioxidant and antimicrobial activity, phenolic or vitamin content, biosynthesis of vitamins, and degradation of anti-nutritive compounds (Szutowska, 2020). All of this contributes to both the functional properties and the flavor development of the various food products in which LAB are present.

1.4.2. *Lactobacillus* as Probiotic

As probiotics, *Lactobacillus* strains have many beneficial effects on health; therefore, they are mainly used to treat or prevent diarrhea, stimulate the immune system, and promote the growth and colonization of beneficial intestinal bacteria (Ren *et al.,* 2014). Over the past decades, *Lactobacilli* as probiotics have been extensively studied, and a wide variety of *Lactobacillus* strains have been selected and used as probiotic bacteria in functional foods as GI biotherapeutics and have been shown to be safe for human use (Minj *et al.,* 2021). Survival and

colonization of probiotic microorganisms in the GI tract are critical properties that confer health benefits to the host (Konstantinov *et al.,* 2008). Some *Lactobacillus* strains require specific cell surface properties such as hydrophobicity and extracellular protein profiles to colonize the gut (Xu *et al.,* 2009). In addition, probiotic strains of *Lactobacillus* must tolerate bile, lysozyme, and low pH (Cele, Nyide, and Khoza 2022). However, although growing evidence showed that *Lactobacilli* are related to these probiotic properties, they are not applicable to other strains without specific functional evaluation (Tulumoglu *et al.,* 2013). They must also have functional properties, such as antioxidant and immune-modulatory activities, to exert beneficial physiological functions (Huang *et al.,* 2016). However, although there is growing evidence that *lactobacilli* are associated with these probiotic properties, they are not applicable to other strains without specific functional evaluation (Tulumoglu *et al.,* 2013).

Water kefir consists mainly of LAB (Waldherr *et al.,* 2010), which synthesize low molecular weight products, mainly organic acids, from sucrose by means of glycosyltransferase enzymes (Pidoux, Brillouet, and Quemener 1988) or together with other carbohydrates (fructose and glucose), which may have antimicrobial properties as they freely diffuse through the cell membrane in their protonated form and lower the intracellular pH by ionizing inside (Chung *et al.,* 2003). The major lactic acid potential probiotic species strains present in WKGs were *Lactobacillus casei/paracasei, Lactobacillus harbinensis, Lactobacillus hilgardii, Bifidobacterium psychraerophilum/crudilactis* Gulitz *et al.,* 201; Laureys *et al.,* 2017). In the case of *L. casei* strains, it is known to possess several functional properties, such as producing antimicrobial compounds, improving intestinal barrier function, reducing pathogen adhesion, and modulating the immune system (Optibac Probiotics Database 2025). Microbial diversity in water kefir fermentations of different origins appears to remain stable between fermentations. On the other hand, many species of lactobacilli are natural inhabitants of the human gastrointestinal tract and form a subdominant genus inhabiting the colon, outnumbered by the dominant species *Bifidobacterium* and *Bacteroides*.

1.4.3. Bifidobacterium as probiotics

Bifidobacteria are microorganisms belonging to the phylum *Actinomycetota* and comprise about 3-7% of the total microbiota of the adult human gut and about 90% in newborns (Cheikhyoussef *et al.,* 2009; Hopkins, Sharp, and Macfarlane 2001; Di Gioia *et al.,* 2014). They are gram-positive, non-spore forming, anaerobic, but some species can tolerate oxygen, non-motile, and catalase-negative bacteria

(Pokusaeva, Fitzgerald, and Van Sinderen 2011). Some *Bifidobacteria* are known to produce antimicrobial substances (Cheikhyoussef *et al.,* 2009; Collado *et al.,* 2005), the activity of which is attributed to the production of acetic acid, lactic acid, and other compounds such as peptides or bacteriocin-like substances (Cheikhyoussef *et al.,* 2009), compounds that have been shown to inhibit the growth of pathogens such as *Listeria monocytogenes*, *Clostridium perfringens*, and *Escherichia coli*. There is still little information available on the antimicrobial compounds produced by strains of this genus; a more detailed evaluation of them could determine the extent to which their production contributes to the probiotic properties of specific *Bifidobacterial* strains and determine their relevance for application in functional foods or pharmaceuticals (Martinez *et al.,* 2013). In fermented WKG, antimicrobial activity has been reported (Barbosa *et al.,* 2011), which has been attributed to the secretion products of these microorganisms (peptides, organic acids or alcohols) (C.A. Caro Vélez and León Peláez 2014; Gamba *et al.,* 2016; Miao *et al.,* 2016; Golowczyc *et al.,* 2007), but the microorganisms responsible for this functional property are not yet known lactic acid bacteria as probiotics.

1.4.4. Yeasts as probiotics

The human gut micro-biome is composed not only of bacterial species, but also of fungal and archaeal species (Chin *et al.,* 2020). Studies of host-microbe interactions have shown that these relationships are complex. An important aspect is the discovery of multiple fungal species involved in human and mammalian intestinal homeostasis, as they are hosted in the intestinal environment where symbiotic benefits to the host are thought to exist. These benefits may exist in the form of immune modulation, adaptive immunity, or general maintenance of intestinal microbial homeostasis through specific interactions (Tsai *et al.,* 2019). Although the most well-characterized probiotic strains microorganisms are bacteria such as *Bifidobacteria* and *Lactobacillus* (Mountzouris *et al.,* 2007), certain yeasts have been shown to have health benefits in several studies (Czerucka, Piche, and Rampal 2007). The most common yeast with proposed probiotic effects is *Saccharomyces boulardii*, also known as *Saccharomyces cerevisiae* var. *boulardii* or *Saccharomyces cerevisiae* Hansen CBS 5926. This yeast is commonly recommended for the treatment of acute GI conditions such as rotavirus and bacterial diarrhea (Kelesidis and Pothoulakis 2011) and chronic conditions such as inflammatory bowel disease (Nielsen, Vainer, and Rask-Madsen 2001).

1.4.5. Dextran from water kefir as a prebiotic polymer

According to the ISAPP 2023 consensus definition, a prebiotic is a substrate that is selectively utilized by host microorganisms to provide health benefit to the host. The exopolysaccharide (EPS) matrix of water kefir makes up these macroscopic grains (Waldherr *et al.,* 2010). Many microorganisms present in food fermentations, such as LAB, can produce some type of EPS that can be classified as functional or prebiotic (Frengova *et al.,* 2002; Barbosa *et al.,* 2011). Prebiotics are ingredients that intervene in microbial activity at the GI level and confer health benefits to the host (Pokusaeva, Fitzgerald, and Van Sinderen 2011; Barbosa *et al.,* 2011; Huang *et al.,* 2016). EPS are indigestible, are not absorbed in the human small intestine, and therefore reach the colon intact, where selective fermentation by beneficial colonic microorganisms, such as *Bifidobacteria* and *Lactobacilli*, separates them from common fibers (Slavin 2013).

The main component of the WKG matrix is dextran (80%) (Horisberger 1969). It has been reported that several bacterial species present in water kefir can produce EPS; species such as *Leuconostoc mesenteroides, Lactobacillus nagelii, Lactobacillus hordei* (Gulitz *et al.,* 2011), *Lactobacillus casei* (Galli *et al.,* 1995), and *Lactobacillus hilgardii* (Waldherr *et al.,* 2010) can form dextrans. Dextrans are high molecular weight homopolysaccharides consisting of glucose molecules linked by alpha-1-6 glycosidic linkages. Branching can occur at positions 2, 3, or 4 (Vettori, Franchetti, and Contiero 2012). The branching ratio and chain length influence the rheological properties of EPS (Ismail and Nampoothiri 2010). Due to its stability and good solubility, dextran is widely used in various industries such as medical, pharmaceutical, food, textile, and chemical (Díaz-Montes 2021). It has been reported that water kefir as a source of dextran is useful for obtaining biodegradable films from the whole grain biomass, which have high homogeneity, no cracks, and high transparency (Coma *et al.,* 2019). Figure 1.5 shows stereoscopic images of water kefir grains, a biomass composed mainly of dextran. Figure 1.6 shows scanning electron microscopy images obtained by Neve and Heller in 2002 (Neve and Heller 2002). This study shows the micro-biome of the grain, which is composed of yeasts and bacteria of different shapes that formed a complex biofilm on the surface of the grain embedded in the dextran matrix. Many *coccobacilli* are present on the surface of the yeast, while rod-shaped bacteria are interspersed among them.

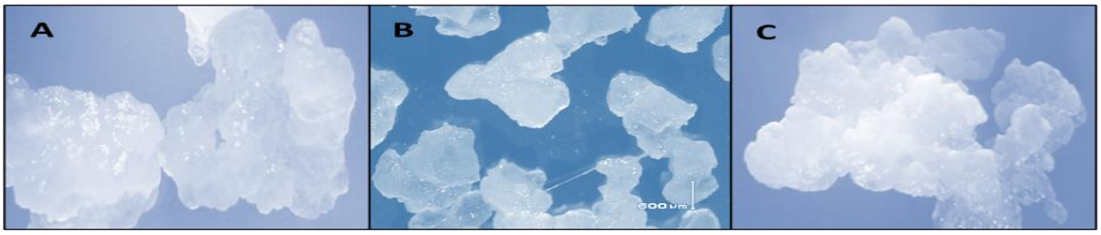

Figure 1.5. *Stereoscopic images of water kefir grains. A and B Colombian water kefir grains; C, Chilean water kefir grains. In the images, the exopolysaccharide dextran can be seen as a gelatinous, translucent material that forms the integument supporting the microorganisms of the WKG microbial community.*

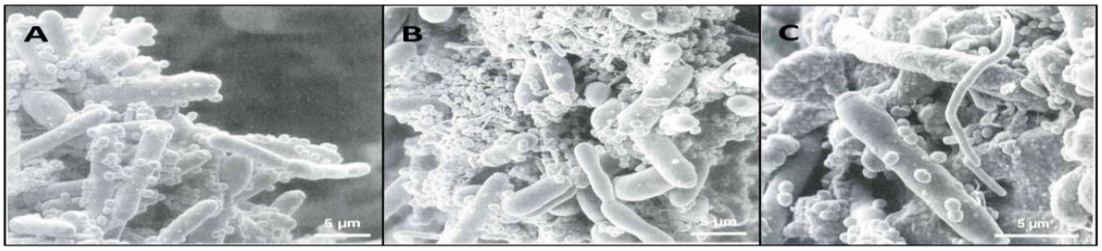

Figure 1.6. *(A y B) Scanning electron micrographs of the microorganisms of WKG with high numbers of yeasts and bacterial cocci (scale bar = 5 um), (B) Scanning electron micrographs showing the diversity of the complex microbiota of water kefir grains consisting of yeasts, cocci, short and long rod-shaped lactobacilli at higher magnification (scale bar = 5 um) (Neve et al, 2002).*

1.5. MICROBIAL DIVERSITY OF WATER KEFIR

As explained above, water kefir contains yeasts, LAB and AAB. Viable yeast counts range from 6.0 × 10^6 to 2.0 x 10^9 cfu/g (colony forming units per gram) of water kefir grains and LAB from 5 x 10^6 to 5 x 10^8 cfu/g (Pidoux 1989; Magalhães *et al.*, 2010; Anna Gulitz *et al.*, 2011), while AAB are underrepresented at 8.5 cfu/g (Galli *et al.*, 1995; Anna Gulitz *et al.*, 2011). In the past, the techniques available to identify microbial diversity were culture-dependent methods and did not fully reveal the microbiota present in this niche. Currently, culture-independent techniques are available, including metabarcoding, which allows for a more in-depth observation of the species that make up the biological systems.

Table1.1 summarizes the different microorganisms found in water kefir in various studies using culture-dependent or culture-independent techniques. The diversity of species found in different water kefir samples from different regions varies widely. Recently, new species such as *Acetobacter sicerae* (Li *et al.*, 2014) and a new species of *Oenococcus* (Verce, De Vuyst, and Weckx 2019) have been discovered in WKG.

Table 1.1. *Microbial groups, genus and species of microorganisms are isolated from WKG reported in various studies.*

Microbial group	Genus	Species	Reference
Bacteria	*Lactobacillus*	*L. brevis, L. buchneri, L. casei subsp. casei, L. casei subsp. rhamnosus, L. paracasei, L. parabuchneri, Lactobacillus kefiri, L. paracasei subsp. tolerans, L. diolivorans, L. casei subsp. Pseudoplantarum, L. fructiovorans, L. collinoides, L. fermentum, L. harbinensis, L. hilgardii, L. hordeii, L. kefiranofaciens, L. kefiri, L. lactis, L. mali, L. nagelli, L. paracasei, L. parafarraginis, L. perolens, L. plantarum, L. satsumensis, L. nagelii/ghanensis, Lactobacillus helveticus, L. satsumensis, L. sunkii, L. ruminis, Bacillus methanolicus, y*	Moinas *et al.,* (1980); Pidoux (1989); (Waldherr, 2010); Galli *et al.,* (1995); Garrote *et al.,* (2001); Simova *et al.,* (2002); (Teixeira *et al.,* (2010); Witthuhn *et al.,* (2005); Chen *et al.,* (2008); Magalha~es *et al.,* (2010); Sabir *et al.,* (2010); Gulitz *et al.,* (2011); Kesmen and Kacmaz (2011); Gulitz *et al.,* (2013); Garofalo *et al.,* (2015); Zanirati *et al.,* (2015); Fiorda *et al.,* (2016a); (Laureys *et al.*,2016); (Laureys *et al.,* 2017); Magalhães *et al.,* (2011); (Yerlikaya *et al.,* 2024)
	Acetobacter	*A. fabarium, A. orientalis, A. lovaniensis*	(Laureys *et al.*,2016); (Gulitz *et al.,* 2013); (Gulitz *et al.,* 2011); (Garofalo *et al.,* 2015); (Magalha~es *et al.,* 2010).
	Lactococcus	*Lcc. lactis supbsp. lactics, Lcc. lactcis supbs. cremoris*	Moinas *et al.,* (1980); (Pidoux, 1989); (Waldherr *et al.,* 2010)
	Leuconostoc	*L. citreum, L. mesenteroides subsp. Mesenteroides, L. mesenteroides subsp. Dextranicum*	Teixeira *et al.,* (2010); Garrote *et al.,* (2001); Magalh~aes *et al.,* (2010); Sabir *et al.,* (2010); Waldherr *et al.,* (2010); Gulitz *et al.,* (2011); Kesmen and Kacmaz (2011); Gulitz *et al.,* (2013); Fiorda *et al.,* (2016a); (Galli *et al.,* 1995; Waldherr *et al.,* 2010); (Pidoux (1989)
	Streptococcus	*Streptococcus lactis, Streptococcus cremoris*	Pidoux (1989)
	Zymomonas	*Zymonmonas sp.*	Marsh *et al.,* (2013b)
	Other species	*Lysinibacillus sphaericus, Oenococcus kitaharae, Oenococcus alcoholitolerans,Oenococcus oeni Bifidobacterium psychraerophilum, Enterobacter hormachei, Gluconobacter frateuri, Bifidobacterium aquikefiri, Bacillus cereus, Gluconobacter liquefaciens, Gluconacetobacter, Gluconobacter sp., Enterococcus faecium.*	Gulitz *et al.,* (2013); Fiorda *et al.,* (2016a); Zanirati *et al.,* (2015); (Waldherr, 2010); (Laureys *et al.*,2017); Marsh *et al.,* (2013); (Verce *et al.,* 2019); (Yerlikaya *et al.,* 2024)

Continuation table 1.1.

Microbial group	Genus	Species	Reference
Yeast	*Saccharomyces*	*S. cerevisiae, S. turicensis, S. bayanus, S. florentinus, S. pretoriensis*	Teixeira *et al.,* (2010); Moinas *et al.,* (1980); Galli *et al.,* (1995); Franzetti *et al.,* 1998); Simova *et al.,* (2002); Wang *et al.,* (2008); Magalha~es *et al.,* (2010); Puerari *et al.,* (2012); Gulitz *et al.,* (2013); Garofalo *et al.,* (2015); Fiorda *et al.,* (2016a), Laureys *et al.,* (2016); (Waldherr, 2010)
	Kluyveromyces	*K. lactis, K. marxianus.*	Ward (1892); Garrote *et al.,* (2001); Teixeira *et al.,* (2010); Wang *et al.,* (2008); Magalh~aes *et al.,* (2010); Magalha~es *et al.,* (2011a); Puerari *et al.,* (2012).
	Lanchancea	*L. fermentati, L. meyercii.*	Magalh~aes *et al.,* (2011a); Magalha~es *et al.,* (2010); Teixeira *et al.,* (2010); Gulitz *et al.,* (2011); Fiorda *et al.,* (2016a).
	Pichia	*P. membranifaciens, P. kudriavzevii, P. cecembensis, P. membranifaciens, P. occidentalis*	Ward (1892); Wang *et al.,* (2008); Fiorda *et al.,* (2016a), Magalhães *et al.,* (2011); Diosma *et al.,* (2014)

1.6. DETERMINATION OF OPTIMAL OPERATING CONDITIONS FOR THE FERMENTATION OF NON-CENTRIFUGAL SUGAR CANE BEVERAGES BASED ON WATER KEFIR GRAINS

Response Surface Methodology (RSM) has been effectively used to optimize and model a wide range of microbial processes. This method combines statistical and mathematical modeling techniques to find the optimal value of variables (Breig and Luti, 2021). Statistical design experiments began in 1951 with Box and Wilson (Box and Wilson, 1951), who applied the idea to industrial experiments and developed RSM. Subsequently, in the 1980s, Genichi Taguchi had a significant impact on popularizing statistical design of experiments and emphasizing its importance for quality improvement (Taguchi, 1987).

In RSM, experimental design data are used to build models that describe the interactions between the independent variables and the responses (dependent parameters). These models are correlations that are used to evaluate the influence of the factors, the interactions with the responses, and the optimization of the process. Typically, the results are described by a 3D plot or a 2D contour plot. The use of RSM as an optimization tool requires many steps, including the selection of the factors, the method of experimentation, the selection of an appropriate model, the verification of the model adequacy, the

representation of the model by a graph, and finally the optimization to obtain the optimal conditions (Breig and Luti, 2021).

RSM optimization requires functions that approximate the solution or the operating point as closely as possible, for which the method of the desirability function is used. This method was proposed by Harrington in 1965 and later improved by Suich and Derringer in 1980. It consists in defining a function in the factor space that measures the Global Desirability (GD) at the predicted points of the response variables, thus transforming the multivariate optimization problem into a univariate optimization problem. The desired operating point is obtained by maximizing the GD. The GD at a point X_0= (X_1, X_2, ..., X_p) is defined as the geometric mean of the individual desirability (d_1, d_2..., dk), which in turn are transformations that convert the predicted values of each response Y_1, Y_2..., Y_k into numbers in the interval [0, 1]. If di equals 1, it means that the corresponding predicted response Y_i takes its maximum desirable value; if d_i = 0, the response takes an unacceptable value and the overall desirability GD = 0, which means that the whole product is unacceptable regardless of the values of the remaining responses (de la Vara and Dominguez, 2000). Functions such as these make RSM a methodology that promises to be a powerful tool for the analysis of variables involved in various biological and other processes.

The organoleptic characteristics of fermented beverages such as NCS water kefir can be influenced by factors such as pH, inoculum and substrate concentration, medium composition, agitation, reactor geometry, dissolved oxygen concentration, and other control variables (Leroi and Courcoux 1996). In fermentation processes with similar microbial communities, as in the case of milk kefir, experimental designs with statistical treatments of RSM have been used to maximize biomass increase and determine the effect of process variables on desired outcomes (Ghasemlou and Khodaiyan 2012). RSM was developed to study the relationships between the responses of different factors in biological systems (Mead and Pike 1975) and to search for the most suitable conditions for a process, helping to minimize costs, maximize products of interest, and increase desired sensory characteristics (Reyes *et al.*, 2005). In addition, RSM helps to describe and identify with great precision the effect of the interactions of different independent variables on the responses when they are varied simultaneously (Hill and Hunter 2012), allowing the optimal levels of the factors to be estimated much more precisely; not only evaluating which of the levels considered is the best, but also finding the exact value that optimizes the design (Fnides *et al.*, 2011). When analyzing an RSM experiment, models are fitted for continuous control variables, often using linear, factorial, or quadratic models such as:

Linear model: $$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \epsilon$$

Factorial model: $$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \epsilon$$

Quadratic model: $$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \epsilon$$

Where: Y is the response variable; β_i are constant terms of the polynomial found by regression, X_i are the control factors and ϵ is a constant. Based on the types of control variables, design software such as Design-Expert recommends that quadratic or factorial models, such as a response surface composite central design (CCD), should be used for continuous process variables (Buxton, 2007).

By controlling factors such as agitation, temperature, available oxygen, inoculum and substrate concentrations in the fermentation of NCS with WKG, it is possible to enhance grain growth, promote microbial groups, increase the production of specific metabolites and organoleptic characteristics, and achieve more standardized fermentations, thereby producing beverages with better sensory and functional quality. (Konopka *et al.,* 2015). One of the main problems in the emerging industry of beverages derived from different varieties of kefir is the standardization of their processing conditions (Dertli and Çon, 2017; Laureys *et al.,* 2017). Methods based on empirical mathematical models based on deep statistical analysis, such as RSM (Gao *et al.,* 2012; Ghasemlou and Khodaiyan, 2012), are proposed to standardize and determine favorable process conditions for WKG fermentation, and to correlate and optimize the system responses with the independent variables under study. (Myers, Khuri, and Carter., 1989). Table 1.2 shows a compilation of fermentation conditions implemented in different varieties of kefir to determine operational ranges for the subject of study of this research.

The growth of water kefir is an important factor for a productive fermentation process, and its increase is related to proper and healthy fermentation. Its growth often slows down, which hinders successful inoculation and prolongs the production process. (Laureys and De Vuyst., 2017b). The low growth rates of WKG are associated with particle sizes below 4 mm, which promote rapid fermentation and high acidification, mainly provided by lactic acid and acetic acid. (Laureys and De Vuyst, 2017b). When this phenomenon of acidification occurs, biomass decreases during the back-slopping process. It has been suggested that this phenomenon potentially affects the activity of glucanase in LAB, thus hindering glucans formation and grain growth. (Kieran M Lynch *et al.,* 2021A growth promoter for WKGs is calcium, due to its buffering capacity, and it is present in tap water, which affects mass gain. (Laureys *et al.,* 2019). Grain growth is a very important variable in this bioprocess, as preservation of the crop is

necessary for subsequent fermentation. (FAO 2010). As mentioned above, it is known that some *Lactobacillus* and *Streptococcus* are responsible for the production of dextran in WKG (Lee *et al.*, 2013), the polymer that makes up more than 80% of it. The morphology and granulometry of WKG is irregular, varying in size and shape, clustering and aggregating to form "larger grains". The size and shape of these grains depend on product handling, metabolic timing and origin. (Fiorda *et al.*, 2017b). See Figures 1.3 and 1.5.

Table 1.2. *Summary of the fermentation conditions (agitation, temperature, oxygen availability, and time) of the kefir varieties that have been evaluated in various studies.*

Kefir Source	Agitation (rpm)	Temperature (°C)	Aerobic/ Anerobic	Fermentation time (h)	Reference
Milk Kefir	nr*	25	Anaerobic and Aerobic	18	Guzel-Seydim *et al.*, 2011
Milk Kefir	0 - 160	25 - 43	nr	24	Zajšek *et al.*, 2013
Milk Kefir	0 - 200	25 - 37	nr	8 - 24	Gao *et al.*, 2012
Milk Kefir	0 - 90	20 - 26	nr	24	Gorsˇek *et al.*, 2007
Milk Kefir	150	28	Aerobic	144	Oliveira *et al.*, 2017
Milk Kefir	nr	20 - 30	nr	nr	Ghasemlou *et al.*, 2012
Milk Kefir	nr	nr	nr	8. - 16	Costa *et al.*, 2019
Milk Kefir	nr	24	nr	18	Atalar *et al.*, 2015
Milk Kefir	0 - 130	18 - 30	nr	24	Schoevers *et al.*, 2003
Water Kefir	nr	25	nr	48	Corona *et al.*, 2016
Water Kefir	nr	21	nr	72	Gulitz *et al.*, 2011
Water Kefir	nr	21	nr	72	Gulitz *et al.*, 2013
Water Kefir	nr	20	nr	24	Hsieh *et al.*, 2012
Water Kefir	nr	21	Anaerobic	192	Laureys et a., 2014
Water Kefir	nr	21	Anaerobic	24 - 120	Laureys et a., 2017
Water Kefir	nr	25	nr	24	Marsh *et al.*, 2013
Water Kefir	nr	26	nr	240	Martínez *et al.*, 2016
Water Kefir	nr	25	nr	24	Teixeira *et al.*, 2010
Water Kefir	nr	21	Aerobic	72	Fels *et al.*, 2018
Water Kefir	nr	22 - 25	nr	24 - 48	Florian *et al.*, 2010

**nr: Not reported*

The microbial species present in the WKG, such as acetic acid bacteria - AAB - which acetify ethanol, are responsible for the spoilage of alcoholic beverages such as wine. (Drysdale and Fleet, 1988). Some authors have reported the presence of AAB in WKG (D. Laureys and De Vuyst, 2014; Fiorda *et al.*, 2017a; Breig and Luti, 2021a), and the presence of acetic acid can lead to undesirable off-flavors in fermented beverages. (Beshkova *et al.*, 2003). On the other hand, the ethanol content in WKG fermenters can vary from 1.1 to 20.3 g/L (David Laureys and De Vuyst, 2014b; Martinez-Torres *et al.*, 2017). This

could be a hurdle, as these types of beverages are often marketed for their health benefits and their significant alcohol content is undesirable. In many countries, the threshold for ethanol content to be labeled as non-alcoholic is 0.5%, but this can vary from country to country. (Bellut and Arendt, 2019). Lactic acid in this fermentation is one of the main metabolites due to the presence of LAB. (Gulitz, 2013; Stadie, Gulitz, Matthias A. Ehrmann, *et al.,* 2013), the species *Lactobacillus paracasei*, *Lactobacillus hilgardii*, and *Lactobacillus nagelii* are the main microbial groups reported in this microbial consortium, and lactic acid is one of the final metabolites that contribute significantly to the flavor of fermented water kefir beverages (Laureys and De Vuyst, 2017b).

The conditions evaluated in other studies for WKG have not considered variations in agitation, and only a few in recent years have studied, using RSM designs, the sensory and metabolic response of fermented formulas based on fruit and/or vegetable substrates such as olives, where the concentrations of inoculum, substrate, time and/or temperature were varied (Plando. And Cimafranca, *et al.,* 2022; Darvishzadeh 2021). Considering the inherent variations in each process, it is necessary to conduct a specific study on the endemic water kefir grains and the native substrates (panela and chancaca) used for these fermentations, which are characteristic of our countries (Colombia and Chile) and make water kefir something unique and representative of the traditions of Latin American culture. On the other hand, finding common points, similarities and trends in the fermentation process of WKG with NCS can provide new insights into the conditions that influence or do not influence this process. Mathematical models that can be used in general to understand the different phenomena in fermentation are valuable because they provide information about behavioral trends despite the changes imposed according to the RSM designs studied.

In this study, temperature ranges and agitation were evaluated under anaerobiosis with known time; inoculum and substrate concentrations to determine if controlling these conditions could produce standardized fermented beverages from WKG.

The ranges for evaluating agitation and temperature in this study were selected from a review of scientific literature related to grains of different types of kefirs from different origins, and the initial inoculum and substrate concentrations were determined in a previous study (unpublished data). Oxygen availability and fermentation time were determined according to the process commonly used in Latin America and the need to avoid off-flavors related to acetic acid, which is formed under aerobic conditions. These operating ranges established for the factors (agitation and temperature) are modulated to obtain optimal values of some physicochemical characteristics of NCS fermented with WKG (ethanol content, reducing

sugars, lactic acid, acetic acid, and biomass) and of the microbiological species involved, values determined according to the food regulations for fermented beverages, sensory profile, and functional and technological potential. Considering all this information, the main objective of this research is to optimize temperature and agitation conditions in the fermentation of non-centrifuged sugar (NCS) using water kefir grains (WKG), through Response Surface Methodology (RSM) and metabarcoding analysis, to establish optimal conditions for producing a fermented beverage with consistent sensory quality and probiotic potential.

1.7. NEXT-GENERATION SEQUENCING (NGS) TECHNIQUES USEFUL FOR IDENTIFYING THE DIVERSITY OF MICROORGANISMS IN WATER KEFIR UNDER CONTROLLED FERMENTATION CONDITIONS.

The term "metabarcoding" involves the collection of environmental samples, DNA extraction, PCR amplification for a gene of interest, and sequencing (Valdez-Moreno *et al.,* 2019); the difference with "metagenomics" is that the latter does not require the physical visualization of the medium sample and involves the collection of amplified and sequenced genes (but without going through PCR, unlike metabarcoding, which does require it). While metagenomics analyzes different nuclear, chloroplast, or mitochondrial sequences without prior use of PCR for sequencing, metabarcoding is based on the same principle but relies on PCR and uses conserved universal sequences with variable regions that can serve as barcodes.

The use of metabarcoding to evaluate the microbial composition of water kefir provides relevant information to determine whether this fermentation process can be standardized at an operational point where microorganisms of interest can be obtained, adding value to the final fermented beverages. As mentioned before, it is necessary to find the optimal operational conditions of the process that influence the microbiological composition of the fermented products, since it is known that this is influenced by the origin of the grain and the substrate used, which is specific to the fermentation characteristic of the Latin American region. Next-generation sequencing (NGS) provides expanded information on the composition of the microbial community, these methods allow for the identification of a greater diversity of microorganisms, including those that are un-cultivable using traditional techniques (Ankit *et al.,* 2021), while culture-dependent techniques only allow us to find microorganisms of interest that can be used as study projections for their application as starter cultures with technological or functional potential. To this study, both methods were used to obtain a broader view of the microbial composition and the possibility of isolating the most prolific species, which will provide information on the dominant

microorganisms. In addition, dependent culture provides microorganisms that can be functionally studied in future research.

1.7.1. Metabarcoding and Response Surface Methodology

Metabarcoding, as a suitable technique for studying the function and composition of a microbial community, has provided information on the relative abundance and diversity of microbial species present in WKG. (Gulitz *et al.*, 2013; Laureys *et al.*, 2017). On the other hand, mathematical models can provide a predictive understanding of consortium behavior in terms of product formation, microbial population growth, biomass formation, and substrate consumption. (Henson and Hanly, 2014). The perspective of using predictive models complements well with genomics, which enhances the understanding of omics, kinetics, and physiological data to better predict and describe the behavior of microbial communities. This allows for the establishment of the most appropriate operating conditions for fermentation, providing information to obtain the expected and desired microbial communities. (Lindemann *et al.*, 2016).

Advances in the ability to monitor the changes of microbial ecosystems include methodological approaches using environmental control, selective pressure, or substrate availability. (Minty et al, 2013). Understanding the need to standardize and understand the fermentations with water kefir grains to achieve non-dairy foods with consistent quality and probiotic potential based on NCS, the use of metabarcoding is proposed as a tool to understand the composition and behavior of the microbiota, which is directly modulated by changes in operational factors (temperature, substrate concentration, agitation, available oxygen). In addition, mathematical models derived from Response Surface Methodology (RSM) are proposed to help predict the optimal operational conditions for obtaining water kefir ferments with significant abundance of desired microbial groups, such as lactic acid bacteria present in WKG, a group of microorganisms of interest for their probiotic potential. In this research, operational points were sought that would also satisfy this condition, along with the improvement of the sensory profile and the proliferation of the grain. This study helps to understand the microbial diversity of WKG through next generation sequencing to identify microorganisms with potential and to obtain information on processing conditions that improve the functional quality of fermented beverages in terms of the abundant presence of lactic acid bacteria.

1.8. IMPORTANCE OF SUBSTRATE CHANGE IN WATER KEFIR FERMENTATION

As mentioned above, in some South American countries, water kefir beverages are traditionally made by placing WKG in a non-centrifuged cane or beet sugar solution with water (Caro Vélez and León Peláez 2014; Caro Vélez and León Peláez 2015; D. Laureys and De Vuyst 2017), while in Europe it is prepared with refined sugar, dried figs (or other dried fruits) and lemon slices. (Anna Gulitz *et al.,* 2011a; Waldherr *et al.,* 2010). Some researchers have developed studies related to WKG with products such as: fermented vegetable and fruit beverages (Corona *et al.,* 2016; Randazzo *et al.,* 2016), soy beverages (Fiorda *et al.,* 2016), bakery products (S. Plessas *et al.,* 2005; Stavros Plessas *et al.,* 2007; Hermann *et al.,* 2016), cheese (Katechaki *et al.,* 2009; Mei *et al.,* 2015), cocoa beverages (Puerari, Magalhães, and Schwan 2012), and beer (Rodrigues *et al.,* 2016).

In recent years, there has been a significant increase in the consumption of foods with functional potential worldwide, which has driven research efforts for the development of non-dairy alternative formats with probiotic potential that can appeal to vegan consumers and those with lactose intolerance. Due to the microbiological and chemical characteristics of water kefir fermented from NCS, it can be stated that it is a food that can potentially lead to the development of lactose-free products, with low sugar content, functional probiotic potential, and free from gluten or other allergens that are currently under scrutiny by scientific associations and consumers seeking to take care of their health through their lifestyle choices.

1.9. TECHNICAL-ECONOMIC PROJECTIONS FOR DEVELOPING A PROTOTYPE FERMENTED WATER KEFIR BEVERAGE WITH PROBIOTIC POTENTIAL

This beverage showed sensory acceptance and as mentioned earlier, has several properties with functional potential that are still under investigation. This thesis project proposes commercializing this product through a market analysis that estimates the economic viability of a prototype of fermented water kefir, flavored with fruit concentrates as flavors, and in a wholesale format that allows reaching the target audience at an affordable cost for individuals, thus providing healthy food of high value. The benefits of this solution are related to the standardization of the beverage production process as a stable product with GRAS (Generally Recognized as Safe) status, allowing consumers (especially those with lactose intolerance) access to nutrient-dense food with a consistently pleasant taste that is economically affordable. Although kefir is made at home, many people stop consuming it despite its benefits because

the fermentation process can be tedious and the lack of control conditions at home results in beverages with variable taste, high acetic acid content, and an unpleasant odor. The product would have a distinctive character, as our native grains have been shown to contain interesting microbial species that can enhance other aspects of health. In addition, the use of NCS as a substrate provides fermented beverages with other functional properties such as antioxidant capacity and significant micronutrient and vitamin content. It is strategic to offer minimally processed food, without additives, which allows the diversification of local product transformations.

1.10. HYPOTHESIS AND OBJETIVES

Hypothesis

The use of Response Surface Methodology (RSM) to optimize temperature and agitation, combined with metabarcoding and physicochemical analyses, can determine the fermentation conditions of non-centrifuged sugars (NCS) with Water Kefir Grains (WKG) that yield a beverage with consistent sensory quality and a characterized microbiota composition, including taxa associated with probiotic potential.

General objective

To optimize temperature and agitation conditions in the fermentation of non-centrifuged sugar (NCS) using water kefir grains (WKG), through Response Surface Methodology (RSM) and metabarcoding analysis, to establish optimal conditions for producing a fermented beverage with consistent sensory quality and probiotic potential.

Specific objectives

- ✓ To obtain individual and global optimal operating points using Response Surface Methodology (RSM), where agitation (rpm) and temperature (°C) are varied, by analyzing observed data of metabolites, biomass, substrate consumption, and microbiological composition obtained from the fermentation of NCS as substrate with water kefir grains.

- ✓ To evaluate the stability of the Water Kefir Grain (WKG) microbial community upon imposition of different substrates and temperature changes by statistical analysis of standard deviations between observed data and using metabarcoding methodology.

- ✓ To evaluate the stability of the microbial community (WKG) and the changes it underwent at the middle and end of the fermentation (48 and 96 hours), to determine the significant differences over time between the populations, and to identify the microbial genera and species present and their respective relative abundances.

- ✓ To evaluate how the change of substrate can influence some physicochemical properties among the evaluated grains from Colombia, Chile, and Germany and to obtain and identify bacteria using culture-dependent methods for future studies of potential probiotic and technological functionality.

- ✓ To propose a project for the development of the NCS fermented beverage with water kefir grains, based on the results obtained in this thesis and through a technical-economic feasibility study.

2. METHODOLOGICAL STRATEGY

As a methodological strategy for this thesis, a staged approach is proposed (see Tables A in Appendix 8.1). In Stage 1, 5 kefir grains from different origins (3 coming from Colombia and 2 coming from Chile) are collected. To select two grains with the best performance in terms of product formation (organic acids, ethanol, and biomass) and substrate consumption, this collection is carried out, and these grains are used in all the studies. The desired ranges of products and sugars are shown in Table A Appendix 8.1, ranges that were established according to the desired sensory characteristics of the final product and the current regulations in Chile for fermented foods. Once the grains were selected, in a second stage, a preliminary identification of the micro-biota present in the two selected WKGs was carried out using a 2^3 factorial design, evaluating two different NCS substrates (panela and chancaca) and two different temperatures (21 and 37°C), with the aim of observing changes in the abundance of genera and microbial species over a fixed period of 96 hours.

In stage 3, a determination of relative abundance and definitive identification of microbial species and genera is performed for both selected grains using a central composite design (CCD) (see design details in Methods and Table A, Appendix 8.1) at 48 and 96 h, using standard deviation as an indicator of significant changes between the time points evaluated. Optimal operating points were also determined using the same central composite design (CCD) with response variables including metabolites (lactic acid, acetic acid, ethanol), biomass formation of kefir grains, sugar consumption (glucose, fructose, sucrose), and abundance of major microbial groups (lactic acid bacteria, acetic acid bacteria, and yeasts) for both grains globally at two different scales (250 and 500 mL).

The two different scales were used to validate whether optimization at the metabolite level varies greatly. In addition, the 500 mL experiment served as a preliminary and observational study so that once optimal conditions were determined, metabarcoding assays could be performed with greater certainty at 250 mL to validate whether temperature and agitation factors caused significant differeces between samples, where it was observed that the fermentation was not reproducible. Therefore, the respective fermentations at 500 and 250 mL were evaluated over a period of 96 hours. The factor ranges (agitation and temperature) selected for the central composite design (CCD) are described in the Methods and Table A, Appendix 8.1, and were chosen based on the literature review. The values to be optimized for the response variables were selected according to the sensory and regulatory criteria described in Stage 1.

In stage 4, the growth capacity of the two selected grains and a grain from Germany (subject of an international internship study) was evaluated, where a substrate exchange was performed between Latin American and European grains (NCS conventional substrate from the Latin American grain and refined sugar as conventional substrate from the German grain; see Figure 3.2 in Methods). The increase in WKG biomass in both the conventional and exchanged substrates was evaluated, and the decrease in pH was observed as an indicator of fermentative power to establish relationships between the degree of acidification and biomass increase. In this phase, an identification of microorganisms was also developed using culture-dependent techniques and MALDI-TOF. In Stage 5, a research project for the development of a fermented NCS beverage with WKG is proposed, based on the results obtained in this thesis and through a technical-economic feasibility study.

3. MATERIALS AND METHODS

3.1. Water Kefir Grain Sample Collection and Inoculum Preservation

Five samples of water kefir grains from different origins and/or locations (Chile and Colombia) were collected by obtaining approximately 100g of each WKG from individuals who maintain a home water kefir fermentation process. Table 3.1 shows the origin of each sample. Among these five collected samples, two of the grains were selected with the aim of obtaining microbial consortia that offered acceptable physicochemical characteristics according to the selection criteria described below in section 3.4 and in Table 3.2 (in terms of acidity, ethanol and sugars) and that showed the best biomass yields of the grains. For the evaluation and selection of the starter cultures, i.e. the different grains of water kefir, preservation processes were carried out in an 8% (w/v) NCS solution at 4 °C. The grains were activated at the same substrate concentration for 72 hours at room temperature without stirring. The WKGs were then sieved, washed with distilled water and reinoculated in an 8% (w/v) NCS solution under the same conditions. This process was repeated three times before the experimental fermentation and until approximately 500 g of each grain sample w s obtained. The NCS used as substrate for the optimization experiments has a composition of 0.28% proteins, 97% carbohydrates and 0.21% minerals, according to the supplier. (Globe Italia - Prodotti Alimentari, Santiago, Chile).

Table 3.1. *Water kefir grains collected from different locations in the countries of Chile and Colombia; this table shows the place of origin and the assigned alphanumeric code for each sample.*

Water kefir grain code	Origin
WKGCOL3	Colombia – Medellín
WKGCOL2	Colombia - Envigado
WKGCOL3	Colombia – Medellín
WKGCH	Chile - Valparaíso
WKGCH2	Chile – Viña del Mar

3.2. Measurement of kefir grains Biomass

Kefir grains were separated from the fermented medium using a plastic sieve (previously sterilized) and then rinsed with a 0.8% saline solution (NaCl). The excess liquid of the sample was removed spontaneously, considering a drainage time of 5 minutes, and then weighed. This procedure was carried out both at the beginning and at the end of the fermentation.

3.3. Fermentation conditions

3.3.1. Fermentation conditions for selecting water kefir grains

Fermentations were performed at 30°C with 8% (w/v) NCS, 8% (w/v) kefir grain inoculum, an air trap system with sodium metabisulfite to create an anaerobic atmosphere, stirring at 130 rpm, at 250 mL volume, and a fermentation time of 96 hours. Each analysis was performed with three biological replicates and three technical replicates. The fermentation conditions are shown in Figure 3.1.

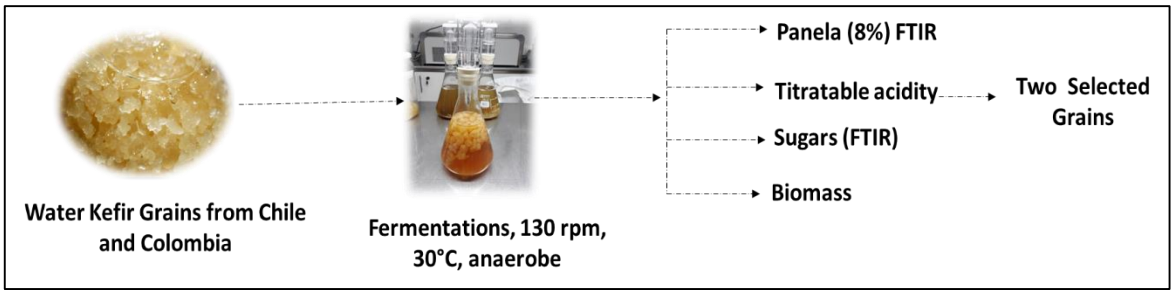

Figure 3.1. *Diagram of the selection process of the kefir grains used in this study, indicating the analyses performed, the number of grains evaluated, the fermentation conditions implemented for each grain, and the quantity of grains selected.*

3.3.2. Fermentation conditions to study optimal operating points of the process using RSM

The two selected kefir grains were reactivated and inoculated in an aqueous solution of Panela at a concentration of 8% (w/v), at 250 mL volume, in a sterile flask under anaerobic conditions. An airlock was used to achieve an airtight seal. The flasks were then placed in an orbital shaker with temperature control according to the experimental design. (Table 3.3). The fermentations were run for 96 hours. The final fermented beverages contained organic acids such as lactic acid and acetic acid, ethanol, carbon dioxide, and sugars (sucrose, glucose, and fructose), which were the products of fermentation by the microorganisms present.

3.3.3. Determination of sugars, titratable acidity, and main metabolites

Determination of total sugars, ethanol, and titratable acidity for water kefir selection

The following determinations were carried out in the Bioreactor and Fermentation Laboratory of the Department of Chemical and Environmental Engineering of the Universidad Técnica Federico Santa María, under the direction of Dr. Alejandra Urtubia. Ethanol and total sugars (sucrose, fructose, glucose) were determined by Fourier transform infrared spectrometry (FTIR). A FTIR spectrometer (4500a, Agilent Technologies) was used to collect mid-infrared spectra. The instrument is equipped with a triple reflection diamond ATR attachment along with a deuterated triglycine sulfate detector and uses an interferometer to scatter the light. Uniform pressure was applied to the samples. FTIR spectra were collected in the 4000-700 cm^{-1} frequency range. The spectral resolution was set to 4 cm -1. Three independent FTIR spectra were collected from each water kefir fermentation sample after 4 days of fermentation. Calculation of sugar consumption (%) is shown in Appendix 8.2, Equation A.

The titratable acidity was determined according to the official AOAC method 942.15 (AOAC 2000). Five grams of fermented water kefir was diluted in 25 ml of distilled water and titrated with sodium hydroxide (NaOH) 0.1N to a pH of 7.0. The titratable acidity was expressed as grams of LA/mL according to equation 2 (see Appendix 8.2, equation B).

Determination of lactic acid, acetic acid, ethanol and sugars to study optimal operating conditions

The HPLC analysis of metabolites and sugars was carried out in the research laboratories of the Department of Chemical and Environmental Engineering of the Universidad Técnica Federico Santa María - Campus San Joaquín, under the direction of Dr. Andrea Carvajal.

The fermented beverage was analyzed after removing the kefir grains with a plastic strainer and using filters for high performance liquid chromatography (HPLC) from Agilent Technologies, model Infinity 1260, under the following conditions: Aminex HPX-87H 300 mm * 7.8 mm, BIORAD, pre-column cation H+, with a mobile phase consisting of H_2SO_4 0.005 mol/L and a flow rate of 0.6 mL/min at 55 °C. Sucrose (SUCR), glucose (GLUC), and fructose (FRUC) were analyzed with a refractive index (RI) detector at 55 °C, while ethanol (EtOH), acetic acid (AA), and lactic acid (LA) were analyzed with a DAD detector at 210 nm. The sample injection volume was 20 µL. The HPLC samples were prepared by taking 1.5 ml of each fermented product diluted in a ratio of 1:3 and then filtered using a sterile syringe attached to a 0.22 um Teflon pore with a diameter of 13 mm; this protocol was taken and modified from (De Sá *et al.*, 2011). Criteria for selecting Water Kefir Grains.

3.4. Criteria for selecting Water Kefir Grains

Based on Table 3.2 and specific criteria according to Chilean technical standards and inoculum requirements, the following objectives are implemented to select two different grains:

- ✓ Kefir Grain Biomass (g/L): The best possible increase of grains to achieve a growth that ensures the maintenance of the starter culture over time.
- ✓ Lactic acid (g/L): Less than 10 g/L to improve the sensory characteristics of the fermented beverage and to prevent excessive acidity that can cause grain death. (Laureys *et al.*, 2017).
- ✓ Ethanol (g/L): The regulatory criterion for potential probiotic beverages is less than 10 g/L by volume. According to the reference of the Sanitary Food Regulation, Decree Number 977/96 of the Ministry of Health of the Republic of Chile.
- ✓ Acetic acid (g/L): As low as possible, the presence of this metabolite in fermented beverages is undesirable. (Drysdale and Fleet, 1988), (Beshkova *et al.*, 2003).
- ✓ Total sugars (g/L): To comply with Chilean Food Labeling Law 20.060, total sugars must be less than 50 g/L.

To apply the criteria described above and to differentiate between the WKGs, a weight or importance was assigned by assigning values of 0, 1 and 2 according to the degree of closeness that the measured parameter achieved with the selection criteria. Table 3.2 shows the description of the weight according to each of the evaluated parameters (biomass increase, acidity, ethanol, total sugar consumption). The calculation of the biomass increase is shown in Appendix 8.2, Equation C.

Table 3.2. *Description of the nominal criterion and the numerical score assigned to evaluate the variables studied (biomass increase, acidity, ethanol, total sugar consumption) in the selection process of two of the five water kefir grains collected.*

Nominal Criteria	Score	Biomass Increase (%)	Acidity g/L	Ethanol (g/L)	Sugar Consumption (%)
Very acceptable	2	> 40	< 5	< 1.5	< 60
Moderately acceptable	1	30 - 40	5 - 7	1.5 - 2.0	60 - 70
Not very acceptable	0	< 30	> 7	> 2	> 70

3.5. Hedonic sensory test

Hedonic test to conduct a preliminary sensory evaluation of the selected grains and identify which of the fermented beverages prepared under the conditions depicted in Figure 3.1, was more highly rated, a sample of 30 untrained individuals was selected to taste the fermented beverage made from both grains. The fermented beverage with the highest acceptance percentage was selected based on the most

positively perceived sensory characteristics. The templates utilized for the sensory acceptance tests are shown in Appendix 8.3, Figures A and B.

3.6. Evaluation of the effect of the operating conditions on the fermentation process by Response Surface Methodology (RSM)

The factors agitation (X_1) and temperature (X_2) were varied under anaerobic conditions and at known inoculum and substrate concentrations, obtaining values for the responses ethanol EtOH (Y_1), lactic acid - LA (Y_2), acetic acid - AA (Y_3), final grain biomass - FG (Y_4), glucose - GLUC (Y_5), fructose - FRUC (Y_6) and sucrose - SUCR. (Y_7). The experiment consisted of a central composite design (CCD) (Mechmeche *et al.,* 2017) where temperature (20 - 40 °C) and agitation (0 - 160 rpm/revolution per minute) were evaluated, with a total of 14 experiments for each of the two selected water kefir grains. The design levels (0-160 rpm) and (20-40 °C) were set according to the state-of-the-art review as shown in Table 1.2.

3.7. Statistical analysis to find optimal operating points

The CCD was designed with eight axial points and six central points. (To ensure reproducibility, a duplicate was established at the central point (see Table 3.3), with $\alpha = 1.414$. The effect of the factors on the responses was evaluated using RSM to find mathematical polynomials describing the composition of the beverage in terms of EtOH, LA, AA, sugars (GLUC, SUCR, and FRUC), and WKG biomass increase, abundance of Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and yeast (LEV), which are used for prediction purposes. The responses were constructed using polynomial regressions of different orders, as described in equation 1 (Mechmeche *et al.,* 2017).

Equation 1. $$Y = b_0 + b_0 X_1 + b_2 X_2 + b_{11} X_1^2 + b_{22} X_2^2 + b_{12} X_1 X_2$$

Where β_0, β_i, β_{ii} and β_{ij} ($i \neq j$) are the regression coefficients. The letter Y denotes an arbitrary response, while X represents the independent variables. (Data analysis was performed using Design-Expert software, version 12. (Stat-Ease Corporation, USA) The lack of fit was assessed using the ANOVA procedure and was considered significant when $p < 0.05$. The operating points for temperature and

stirring were identified by applying an optimization algorithm using a software function that allows different levels of constraint satisfaction for minimization and maximization problems, the Global Desirability (GD) function, to assess the reliability of the operating point obtained. (Derringer & Suich, 1980).

Table 3.3. *Levels of raw and coded values for the agitation and temperature factors chosen to perform fermentation experiments according to the CCD design.*

Run	**Factor Levels**		**Encoding Factors**	
	Agitation rpm	Temperature T(°C)	X_1 Agitation	X_2 Temperature
1	136,57	22,93	1	-1
2	160,00	30,00	+α	0
3	136,57	37,07	1	1
4	80,00	40,00	0	+α
5	23,43	37,07	-1	1
6	0,00	30,00	-α	0
7	23,43	22,93	-1	-1
8	80,00	20,00	0	-α
9	80,00	30,00	0	0
10	80,00	30,00	0	0
11	80,00	30,00	0	0
12	80,00	30,00	0	0
13	80,00	30,00	0	0
14	80,00	30,00	0	0

The values of the levels of the independent variables (stirring and temperature) were analyzed using the desirability function method with Design Expert software version 12.0.0. (Statease Inc., Minneapolis, USA). The optimal operating conditions for WKGs were determined according to the specific criteria described in section 2.4.

3.8. Preservation and Activation of Water Kefir Grains for Metabarcoding Study

The starter culture, i.e. WKG, was washed with a sterile saline solution (NaCl) at 0.8% (w/v). To preserve the grains, they were added to a sterile aqueous solution of 5% NCS at 4°C. Activation of the grains was carried out without agitation, at room temperature for 72 hours, using the substrate NCS (8%) and at concentrations of WKG at 8% (w/v). The grains were then sieved, washed with sterile saline and reinoculated with 8% (w/v) NCS solution. This process was repeated three times until 800 g of each of the two selected grains were complete.

3.9. Metabarcoding study of water kefir

3.9.1. Preliminary metabarcoding study

As a preliminary test, an experiment was conducted with two different NCS substrates to observe the variability in the relative abundance of the microbial community in the two selected grains (WKGCOL3 and WKGCH2). The selected temperatures and substrates were determined according to the conditions described in Table 1.2. Activated WKGs were inoculated at a concentration of 8% (w/v) in aqueous solutions of Panela and Chancaca at 8% (w/v) in 250 mL sterile flasks, under anaerobiosis and without agitation. For this purpose, an airlock was used to achieve a hermetic seal. The design experiment is described below in Table 3.4.

3.9.2. Statistical analysis in preliminary metabarcoding study to assess substrate change and temperature

A 2^3 factorial design was used to determine whether there were significant differences between the samples and whether the changes in substrate and temperature were significant for each grain. The design is a non-block factorial with 8 replications without replications at the center, type 3FI (3 interaction factors—2 categorical and one nominal) and two levels. The response variables studied were lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeasts (LEV).

Table 3.4. *The following table describes the experimental design performed for the preliminary analysis of the metabarcoding of the two selected kefir grains subjected to changes in substrate and temperature. The categorical factors (substrate and grain) are shown along with the numerical factors (temperature). The substrate categorical factor is represented by the words "PAN" (panela-cane sugar) and/or "CH" (chancaca-beet sugar), which denote the two substrates used. The next categorical factor is represented by "COL" (Colombia) and/or "CH2" (Chile), which indicate the origin of the grains, and finally "21" and "37" indicate the temperatures, which represent the numerical factor. The coded values of the factors are also shown.*

Substrate (g/L) Factor 1	Temperature (°C) Factor 2	Grain Factor 3	Encoding Factor 1	Encoding Factor 2	Encoding Factor 3
PAN	37	CH2	{1}	1.000	{-1}
CH	37	CH2	{-1}	1.000	{-1}
PAN	21	CH2	{1}	-1.000	{-1}
PAN	21	COL3	{1}	-1.000	{1}
CH	37	COL3	{-1}	1.000	{1}
PAN	37	COL3	{1}	1.000	{1}
CH	21	COL3	{-1}	-1.000	{1}
CH	21	CH2	{-1}	-1.000	{-1}

3.9.3. Metabarcoding study to investigate the impact of different operating conditions on the variation of microbial composition.

A metabarcoding study was conducted to investigate the impact of varying operational conditions on microbial communities. The microbial composition was evaluated at the genus and species levels in accordance with the previously designed experiment, which was conducted to select the optimal operational fermentation conditions (CCD) (see Table 3.3). The analysis was conducted at 48 and 96 hours in duplicate at each time point for both selected grains. (WKGCOL3 and WKGCH2).

3.9.4. DNA extraction for metabarcoding analysis of water kefir

The DNA extraction method was carried out according to the method recommended by the supplier (DNeasy Power Soil Kit - QIAGEN - 2016), which was satisfactory in terms of the quality of the extracted genetic material. Table A in Appendix 8.4 shows the measurements indicating the quality of the DNA. The 260/280 ratio is very stable, and it is considered that DNA of optimal purity has a value between 1.8 and 2.0. In summary, the extraction protocol was next:

Next, 250 mg of kefir grains and 800 µl of CD1 solution were added. These were vortexed briefly in a vortex tube and then shaken at maximum speed in FastPrep 24 for 10 min, followed by centrifugation at 30,000 × g for 1 min. The supernatant was transferred to a clean microcentrifuge tube and 200 µl of CD2 solution was added. It was centrifuged again at 30,000 × g for 1 min, avoiding the precipitation, and up to 700 µl of the supernatant was transferred. The supernatant was transferred to a clean 2 ml microcentrifuge tube. 600 µl CD3 solution was added and shaken for 5 seconds. 650 µl of lysate was applied to an MB centrifugation column. It was centrifuged at 30,000 x g for 1 min and the flow was discarded. The lysate was then repeated with 650 µl of CD3 solution to ensure that all the lysates had passed through the MB spin column. The MB spin column was carefully placed in a clean 2 ml collection tube, taking care not to splash the column with the lysate flow. Next, 500 µl of EA solution was added to the MB spin column and centrifuged at 30,000 x g for 1 min. The flow-through was discarded and the MB Spin Column was returned to the same 2 ml collection tube. 500 µl of C5 Solution was added to the MB Spin Column and centrifuged at 30,000 x g for 1 minute. The flow-through was discarded and the MB Spin Column was placed into a new 2 ml collection tube and centrifuged at 30,000 x g for 2 min. The MB Spin Column was carefully placed into a new 1.5 ml elution tube. 50-100 µl of Solution C6 was

added to the center of the white filter membrane and centrifuged at 15,000 x g for 1 min. The MB spin column was discarded. The DNA is now ready for downstream applications.

3.9.5. Massive sequencing of water kefir grains and Bioinformatics methods

The hypervariable regions V1-V2 of the bacterial 16S ribosomal RNA gene were amplified with primers 16SF_Bif_BK_ACGACGCTCTTCCGATCTAGRGTTHGATYMTGGCTCAG and 16SR_Bif_BK_GACGTGTGCTCTTCCGATCTTGCTGCCTCCCGTAGGAGT. For fungal species, the ITS1 and ITS2 regions were included with primers ITS1f_Illu_ACGACGCTCTTCCGATCTCTTGGTCATTTAGAGGAAGTAA and ITS2r_Illu_GACGTGTGCTCTTCCGATCTGCTGCGTTCTTCATCGATGC. PCR amplification was performed using Bio-Rad reagents and a Bio-Rad CFX96 Real-Time System C1000 thermocycler. DNA integrity was confirmed by agarose gel electrophoresis. The PCR products were purified and used to prepare six DNA libraries for ILLUMINA MiSeq sequencing using the TruSeq DNA Library Protocol. Between 1.4 and 2.4 million base pairs were sequenced for the library (the experiments for all metabarcoding analyses were performed at the Helmholtz Center for Infection HZI, Braunschweig - Germany), under the direction of Dr. Dietmar Pieper).

Based on the samples analyzed via MiSeq Illumina, the fastq files were analyzed and the phyloseq was generated based on the pipeline of the DADA2 package version 1.12.1 in R. The direct and reverse reads were trimmed to 20 and 19 bases, respectively, and truncated to a length of 240 bases. A maximum of two expected replicates per read was allowed. Sequence variants that accumulated ≤2 reads were restricted, and the remaining variants were annotated based on Bayesian classification with an 80% pseudo-bootstrap threshold using the RDP 18 set for both arcup ITS and Unite ITS. (Dos Anjos-Borges *et al.,* 2023; Orberste *et al.,* 2024). Bacterial and fungal sequences were obtained. Samples generating less than 10,000 sequence reads were discarded. Sequence variants were then manually analyzed against the RDP database using the Seqmatch function. Species-level annotations were assigned to a sequence variant only fragments of the 16S rRNA gene from a single species aligned with a maximum of two mismatches. In total, just over 7 million sequence reads were generated for fungi and more than 9 million for bacteria. The data were rarefied using the rarecurve function in R (Orberste *et al.,* 2023). The reads were processed using "CutAdapt" to trim primers and adapters. The R package "DADA2" was used to filter reads by quality (dereplication and chimeras) and size (minimum 140 bp) and to merge paired reads. Taxonomic annotation of fungi was performed using the Warcup Fungal ITS database and the

Unite Fungal ITS database (specific for ITS) in the RDP classifier. Sequences were also compared using BLAST with verified bacterial taxonomic annotation and further verified using RDP, assigning a species name only if the similarity core was >0.95 and only for sequences from a single species.

Molecular identification was performed using the databases and a third annotation was performed using Blast, including the abundance of each of the 46 annotated fungal phylotypes and the 77 bacterial phylotypes for each of the samples. The identified filotypes were filtered based on average abundance (> 0.001%) and sequence direction (reverse complement). The final annotation was based on the combination of the above databases. The species of microorganisms were identified, with very close results according to the database used for their identification.

3.10. Water kefir grain samples and Inoculum preservation for substrate change experiments.

Three samples of WKG were collected from different countries of origin (Chile, Colombia, and Germany), two of which corresponded to the two previously selected WKGs, for about 100 g of WKGs. Table 3.5 shows the origin of each sample. The grains WKGCOL3 and WKGCH2 from Latin America were preserved in a 6% (w/v) NSC solution at 4 °C, and the grains from Germany (WKGALE) were also preserved at 4 °C in a 6% (w/v) refined sugar solution containing two dried figs (substrate typically used in Freising - Germany for the fermentation of water kefir grains). The activation of the grains was carried out under the same conditions, without agitation, at room temperature for 72 hours; this process was repeated three times until approximately 600 g of each grain sample was obtained. The unrefined sugar used as substrate for the experiments was provided by Globe Italia - Food Products, Santiago, Chile. The substrate change experiments were performed at the Department of Technical Microbiology, Weihenstephan School of Life Sciences, Technical University of Munich - TUM, Germany, under the supervision of Prof. Dr. Rudi F. Vogel.

Table 3.5. *The following table describes the sample code used to evaluate the substrate change. The code for each sample was constructed using the words "WKG" for water kefir grains, "ALE" for the grain of German origin."COL3" for the selected grain of Colombian origin, and "CH2" for the selected grain of Chilean origin.*

Water kefir grain code	Origin
WKGALE	Germany – Freising
WKGCOL3	Colombia – Medellín
WKGCH2	Chile – Viña del Mar

3.10.1. Fermentation conditions and substrate change

The fermentations were carried out in two different experiments. In the first experiment, eight consecutive steps or fermentations were carried out; the first two were carried out on the conventional substrate used according to the origin of each sample. In the case of WKGCOL3 and WKGCH2, the first two steps were carried out in NCS, and in the case of WKGALE, in sugar with figs. The following six steps were performed by changing the substrates, i.e. refined sugar with dried figs was used for WKGCOL3 and WKGCH2, while NCS was used for WKGALE. The substrate change process is described in Figure 3.2 below. The second experiment was conducted with the remaining grains from experiment 1 in three steps or sequential fermentations. In the first step, a conventional substrate was used, and in the following two steps, a substrate change was performed as described in the first experiment. All the fermentations were carried out at room temperature, under aerobic conditions and without agitation, in properly disinfected glass containers of 435 ml, covered with dry linen, with inoculum concentrations of 30% (w/v) and substrate (sugars) of 6% (w/v), in the case of fermentations in the conventional European substrate. The fermentation time was 48 h.

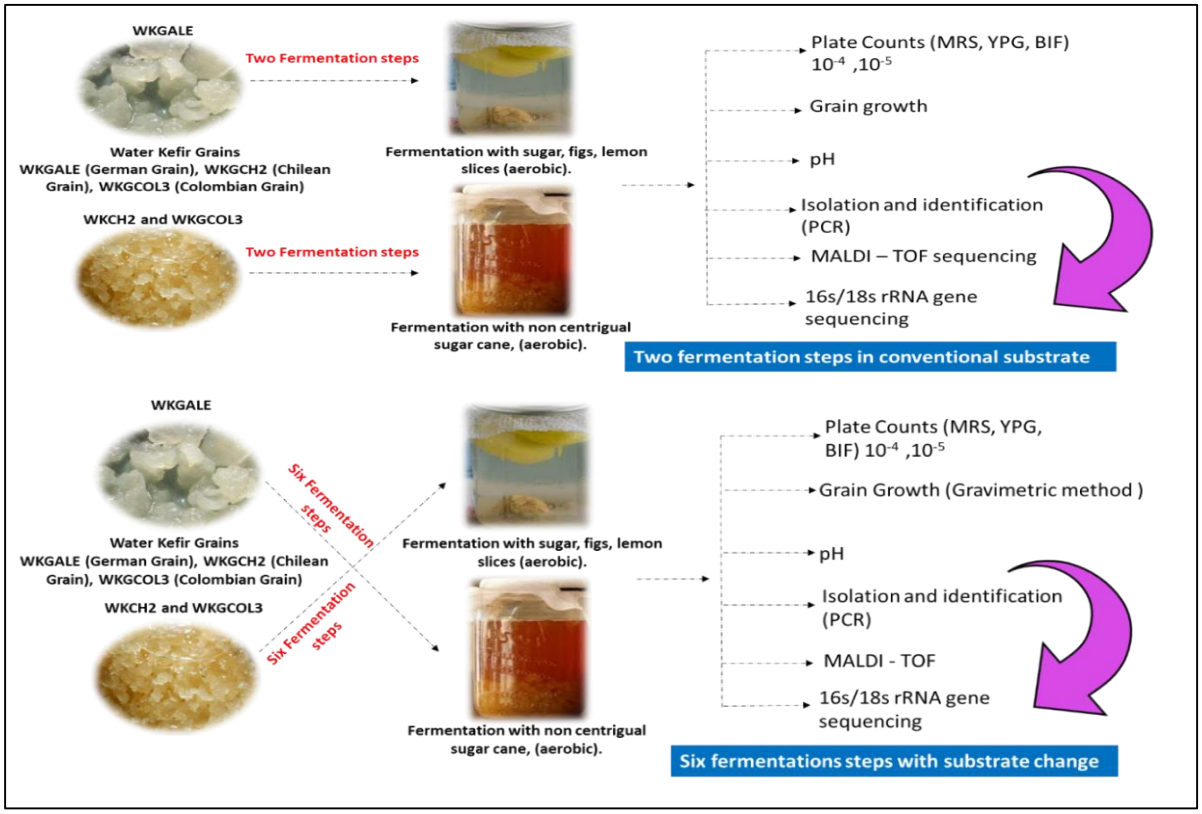

Figure 3.2. *Description of the substrate change experiment. The first two steps correspond to the conventional substrates used for each grain and are shown at the top; the analyses carried out after 48 hours of fermentation are also shown. The bottom part shows the six subsequent steps in which the substrate was changed.*

3.10.2. Measurement of Kefir Grain Biomass and pH Changes

Kefir grains were separated from the fermented medium with a plastic sieve and then rinsed with a 0.8% sodium chloride (NaCl) solution (NaCl). The excess liquid from the sample was removed spontaneously, allowing a drainage time of 5 minutes, and then weighed. This procedure was repeated at the beginning and at the end of each fermentation. As previously mentioned, all fermentation steps were initiated with a 30% (w/v) inoculum, which weighed approximately 261 g of kefir grains, washed with sterile water, and weighed on an electronic balance with an accuracy of ± 0.01 g. The pH of the fermentation medium was measured at 0 and 48 h of fermentation using a pH meter at 25°C, and the pH of the water kefir liquor was measured using a SenTix 41 glass electrode (WTW GmbH, Weilheim, Germany).

3.10.3. Media and microorganisms' growth

For the cultivation of microorganisms, serial dilutions were made by taking 1 ml of the supernatant at the end of the specified fermentation time and adding it to 9 ml of sterile saline solution. The different serial dilutions were inoculated on MRS agar plates containing 2 g/L meat extract, 4 g/L yeast extract, 10 g/L casein peptones, 1 mL Tween-80, 2. 5 g/L $K_2HPO_4x3H_2O$, 5 g/L sodium acetate, 2 g/L diammonium hydrogen citrate, 0.2 g/L magnesium sulfate heptahydrate, 0.038 g/L manganese sulfate monohydrate, 20 g/L glucose, and 15 g/L agar. The pH was adjusted to 5.7, and the glucose was sterilized separately in an autoclave. Cycloheximide (150 μg/mL) was added to the medium after the agar was cooled to 50°C. The MRS agar plates were incubated anaerobically at 30°C for 3 days. For the cultivation of molds and yeasts, serial dilutions were plated on YPG agar plates containing 10 g/L casein peptide, 5 g/L yeast extract, 15 g/L agar, and 20 g/L glucose. The pH was adjusted to 6.5 and the glucose solution was sterilized separately in an autoclave. The agar plates were incubated for 3 days at room temperature. After incubation, viable cell counts were calculated. Individual bacterial colonies with distinct morphological appearance were randomly selected to isolate different microorganisms and cultured on solid MRS and YPG media for 2-5 days at 30°C for subsequent identification by 16s rRNA gene sequencing and/or MALDI-TOF. The cultivation methods described above were performed according to the procedure described by Gulitz in 2011.

For the specific cultivation of *Bifidobacteria*, the same serial dilutions were used as before. The serial dilutions were inoculated on modified tryptone soy agar (mTY) plates which contained 10 g/l casein peptide, 10 g/l soy peptide, 6 g/l NaCl, 5 g/l K2HPO4, 2 g/l glucose, 2 g/l raftilose (ORAFTI, Active

Ingredients for Food, Belgium, Oreye) and 15 g/l agar. The pH was adjusted to 6.1, and after cooling the agar to 50ºC, 0.1 g/L ciclopirox, 0.05 g/L kanamycin sulfate, 0.05 g/L mupirocin, and 0.005 g/L amphotericin B were added. The agar plates were incubated anaerobically (AnaeroGen; Thermo Fisher Scientific - 70% N_2 and 30% CO_2) at 30°C for 3-5 days. Colonies were randomly selected from the *Bifidobacterial* plate cultures and grown in liquid MRS medium for 3 days at 30°C for subsequent identification by 16s rRNA gene sequencing and MALDI-TOF.

3.10.4. Microbial Analysis by MALDI-TOF

To determine the composition of the microbiota using MALDI-TOF MS, colonies were collected from each plate. If there were fewer than 96 colonies on a plate, all colonies were collected for identification; if the plates contained more than 200 colonies, all colonies growing in one quarter of the plate were collected for identification. Colonies were seeded directly onto a stainless steel MALDI plate (Bruker Daltonics, Bremen, Germany) and covered with 1 μL formic acid (FA) for protein extraction. After drying in the extraction hood, the sample was covered with a matrix solution containing 10 mg/mL alpha-cyano-4-hydroxycinnamic acid in acetonitrile, deionized water, and trifluoroacetic acid (50:47.5:2.5, v/v) and allowed to dry in the extraction cabinet.

Mass spectra were generated using a Microflex LT MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser ($\lambda = 337$ nm) at a laser frequency of 60 Hz, operating in linear positive ion detection mode under real-time classification (RTC) of MALDI Biotyper 3.0 (Bruker Daltonics, Bremen, Germany) and FlexControl 3.4 (Bruker Daltonics, Bremen, Germany). The data were processed as described in Kern *et al.*, 2013 and Usbeck *et al.*, 2013, and the total number of reproducible peaks within the replicated raw spectra was determined using a calculation method based on LIMPIC (Linear Ion Peak Indication and Classification for MALDI-TOF MS), in which the peak selection parameters were set to a signal-to-noise ratio of 3 and a peak width of 0.5 Da. Therefore, a total of 30 individual spectra per strain and method were merged into a single spectrum using LIMPIC. The minimum acceptable peak detection rate, which refers to the ratio of the number of spectra containing a given peak to the total number of spectra analyzed, was set at 0.8 to allow only those peaks present in at least 80% of the individual spectra to be included in the merged spectrum. Subsequently, all peaks with an intensity of 1000 arbitrary units (a.u.) or more were counted, reaching an effective range of up to approximately 5 × 10^4 a.u., in the mass range between 3000 and 12,000 Da, and assigned to five intervals based on their intensity. Sample identification was performed using MALDI BioTyper 3.0 software (Bruker Daltonics, Bremen, Germany), which includes a reference database of 4,111 microbial spectra, of which 242 belong to the genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc*, covering

approximately 100 different species. A logarithmic score >2.0 indicated successful identification of a sample at the species level according to the manufacturer's instructions. Clin ProTools 2.2 (Bruker Daltonics, Bremen, Germany).

3.10.5. DNA Extraction for Bacteria Identification

Bacteria grown in MRS medium were isolated and re-grown in liquid MRS medium for 3 days at 30°C in anaerobiosis; 2 ml of the bacteria grown and cultured in the medium were then collected and centrifuged at 13,000 rpm for 30 seconds. The supernatant was discarded, and the precipitate was washed with 1 ml TE buffer containing 1 mM EDTA and 10 mM Tris at pH 8 and centrifuged again. Bacterial DNA was isolated using the Bacterial DNA Kit (D3350-02, OMEGA bio-tek, USA) according to the instructions. The precipitate from each bacterium was resuspended in 200 µL TE buffer containing lysozyme (10 mg/ml) and the mixture was incubated at 37°C for 60 min. DNA was eluted twice with 50 µL elution buffer.

3.10.6. Amplification and Sequencing

With the aim of obtaining a bank of microbial strains to evaluate their probiotic capabilities and technological potential in the future, 6 strains were selected from a total of 32 lactic acid bacteria collected. The criteria for the selection of these strains were based on the interest in identifying novel microorganisms that had not been previously reported, selected according to their morphology, culture conditions and microscopic visualization, with a preference for potential strains from the genera *Bifidobacterium* and *Oenococcus.* DNA was isolated from the six selected microorganisms and the V1 to V4 regions of the bacterial 16S rRNA gene were amplified using primers Ba27f and Ba519r. The reaction mixture consisted of 0.1 mmol/l of each deoxyribonucleoside triphosphate, 0.75 U of Taq polymerase, 5 pmol of each primer, and 1 µl of genomic DNA, and amplification was performed using a PCR program of 94°C for 2 min; 32 cycles of 94°C for 45 s, 52 °C for 90 s, and 72°C for 2 min; and a final step at 72°C for 5 min. The quality of the PCR amplified products (approximately 520 nt in length) was confirmed by electrophoresis, and the products were purified using a Cycle Purification Kit (Omega Bio-Tek) according to the manufacturer's instructions. A sample of 20 µl of each amplicon was sequenced on a 454 FLX Titanium genome sequencer from LGC Genomics (Berlin, Germany). (Weisburg *et al.,* 1991; A. Gulitz *et al.,* 2013).

3.10.7. Species identification

The amplified fragments were verified using sequencing by Eurofins Genomics (Ebersberg, Germany) and comparative searches using the Basic Local Alignment Search Tool (BLAST) in the GenBank/EMBL/DDBJ database. Amplified fragments were verified by sequencing by Eurofins Genomics (Ebersberg, Germany) and comparative searches using the Basic Local Alignment Search Tool (BLAST) in the GenBank/EMBL/DDBJ database.

3.11. Feasibility proposal for the development of a marketable prototype water kefir beverage based on NCS.

Activity 1: Following the optimal operating point found in this research, validate it at volumes up to 10 liters.

Activity 2: Understand microbial kinetics to determine operating time at larger process volumes.

Activity 3: Levels of viable microorganisms in the final product.

Activity 4: Probiotic abilities of microorganisms.

Activity 5: Obtain prototypes of flavored and natural beverages using traditional fruits and plants from our regions.

Activity 6: Sensory profile of prototype beverages.

Activity 7: Packaging tests in containers made of different materials with sustainable or reusable characteristics. Determination of usable volume and use of headspace gases. Stability tests during shelf life.

Activity 8: Sensory tests with users at strategic points according to market analysis.

Activity 9: Patentability study, evaluation of intellectual property strategy, and registration of trademarks and trade secrets.

Activity 10: Formalization of the legal entity.

4. RESULTS AND DISCUSSION

4.1. Selecting water kefir grains

The growth of kefir grains in this type of fermentation is important because the starter culture relies on the reuse of filtered grains from previous fermentations. (back-sloping). (FAO 2010). The WKGs showed an increase in biomass ranging from 16.0% to 55.8%, considering that this is a percentage increase in relation to the final weight. In terms of acidity, values between 5.37 and 8.08 g/L of titratable acidity expressed as lactic acid, a sugar consumption between 56.2 and 73.3% and an ethanol production between 1.17 and 2.28 g/L were found. Based on the results obtained, heterogeneity was observed in the growth of the grains and in the formation of the main products. Table 4.1 shows the measured values for each parameter evaluated in the five varieties of WKG.

Table 4.1. *Measured values for each evaluated parameter (final biomass, acidity, ethanol, and total sugars) in the five varieties of water kefir grains from different origins (Chile and Colombia).*

Kefir Grain Code	Final Biomass (g/L)	Biomass increase (%)	Acidity (g/L)	Ethanol (g/L)	Total Sugars (g/L)	Sugar Consumption (%)
WKGCOL	64.83 ± 5.23	38.30	8.08 ± 0.35	2.28 ± 0.07	30.08 ± 2.82	62.40
WKGCOL2	63.65 ± 1.83	37.16	6.75 ± 0.48	2.22 ± 0.26	21.39 ± 1.89	73.26
WKGCOL3	73.88 ± 7.87	45.86	5.11 ± 0.67	2.06 ± 0.29	25.20 ± 1.93	68.50
WKGCH	47.62 ± 1.82	16.00	5.37 ± 0.59	1.79 ± 0.26	35.02 ± 7.17	56.23
WKGCH2	90.58 ± 6.22	55.84	4.83 ± 0.41	1.17 ± 0.06	26.46 ± 2.21	66.93

According to the weighting proposed in the methodology, a total score is obtained for each of the kefir grains. Table A shows in Appendix 8.5 the score assigned to each of the evaluated parameters.

As can be seen, the highest scores were obtained by the water kefir grains WKGCH2 and WKGCOL3 with 7 and 5 points respectively, which were selected as candidate grains for useful starter cultures to produce standard fermented beverages. Based on the results obtained, heterogeneity was observed in the growth parameters and in the formation of primary metabolites for the different grains. Figure 4.1 shows the classification of WKG, where the grain WKGCH2 has the best fermentative potential, while the grains WKGCH and WKGCOL did not exhibit good growth or suitable fermentation characteristics. These results are useful for identifying candidate grains (WKGCOL3 and WKGCH2) for subsequent kinetic studies and metabarcoding that will enable the development of new starter cultures for the probiotic industry.

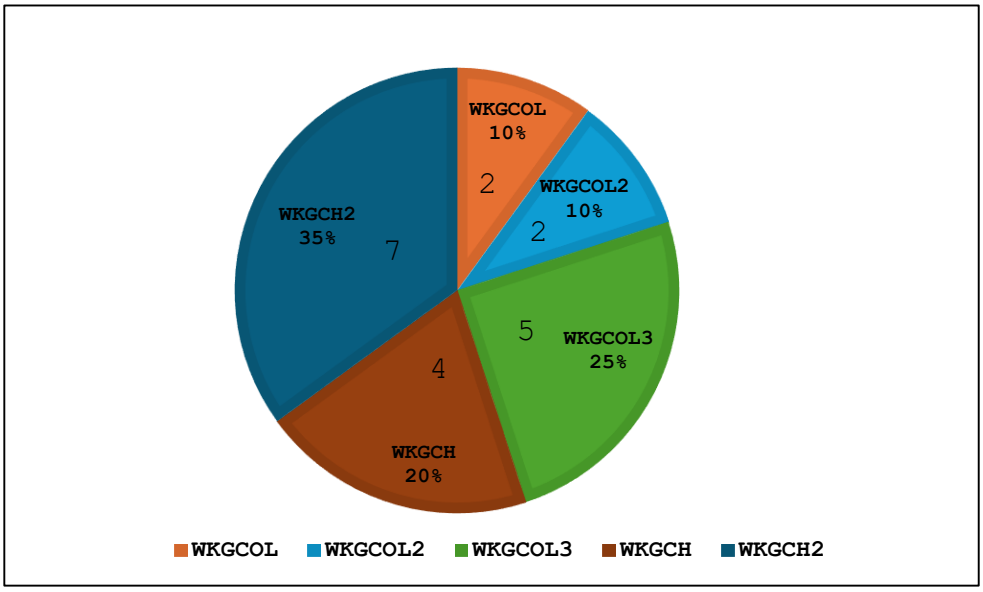

Figure 4.1. *Total score assigned according to what is described in Section 2.4 of the methodology, calculated by summing the score (ranging from 0 to 2) of all evaluated parameters. Each kefir grain was evaluated according to the selection criteria for biomass increase, acidity, ethanol content, and sugar consumption. The numbers and percentages in the figure indicate the total score evaluated.*

4.2. Hedonic sensory test

The acceptance or non-acceptance of the fermented beverage produced with WKGCOL3 and WKGCH2 was determined according to a hedonic test (see Appendix 8.3, Figures A and B), and the following results were obtained. The values described in Tables 4.2 and 4.3 correspond to the percentage of tasters who found the descriptor to be intense on a scale of 0 to 5, with 5 points being the most intense.

Table 4.2. *Results of the sensory analysis of fermentation for WKGCOL3 (sample with code 1654), where 8 flavor descriptors (sweet, acid, bitter, fermented, fruity, astringent, spicy and metallic) are weighted on a scale of 0 to 5. The score given represents the percentage of judges who gave the same rating.*

Descriptor	Percentage of perception (%)					
	0	1	2	3	4	5
Sweet			50	16,7	16,7	16,7
Acid	33,3	16,7	33,3		16,7	
Bitter	83,3				16,7	
Fermented		16,7	33,3	16,7		33,3
Fruity			20	40		40
Astringent	16,7	50,0		16,7	16,7	
Spicy	100					
Metallic	100					

Table 4.3. *Results of sensory analysis of fermentation for WKGCH2 (sample with code 5937), where 8 flavor descriptors (sweet, acid, bitter, fermented, fruity, astringent, spicy and metallic) are weighted on a scale of 0 to 5. The score given represents the percentage of judges who gave the same rating.*

Descriptor	Percentage of perception (%)					
	0	1	2	3	4	5
Sweet				33,3	50,0	16,7
Acid	16,7	33,3	50,0			
Bitter	66,7	16,7	16,7			
Fermented		60,0			20,0	20,0
Fruity			33,3	16,7	16,7	16,7
Astringent	50,0	16,7	16,7		16,7	
Spicy	66,7	33,3				
Metallic	66,7	33,3				

WKGCOL3, N°1654: 50% of the sensory panel found the beverage moderately sweet on a scale of 2, 33.3% found it moderately acid on a scale of 2, 67% found it significantly fermented on a scale of 2 and 5, 80% found it very fruity on a scale of 3 and 5, and 50% found it slightly astringent on a scale of 1. 100% of the sensory panel found no bitter or spicy flavor on a scale of 0.

WKGCH2, N°5937: 50% of the sensory panel found the beverage noticeably sweet on a scale of 4, 50% found it moderately acidic on a scale of 2, 60% of the sensory panel perceived a slightly fermented flavor on a scale of 1, 33.3% perceived a moderately fruity flavor with a score of 2, and 100% of the sensory panel did not perceive any spicy, astringent, bitter or metallic flavors, scoring them 0.

The acceptance and taste perception were conducted using a paired presence/absence test (Appendix 8.3, Figure B). Sixty percent preferred the taste of the WKGCOL3 beverage, thirty percent preferred the WKGCH2 beverage, and twenty percent found no difference.

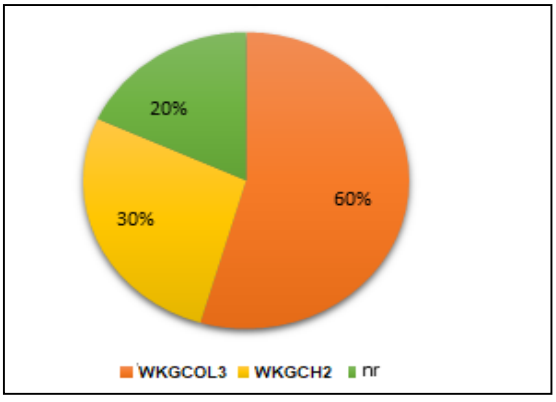

Figure 4.2. *A pie chart showing the percentage distribution of the preference test between WKGCOL3 and WKGCH2. The highest preference percentage was 60% for WKGCOL3.nr (not reported).*

4.3. Preliminary Metabarcoding Study of Water Kefir Grains. (Bacterial composition)

As mentioned above, WKGs are mainly composed of BAL, AAB, and LEV, including the genera *Gluconobacter* and *Oenococcus*, as reported in recent publications. (David Laureys *et al.,* 2017; Verce, De Vuyst, and Weckx 2020a; Gulitz *et al.,* 2011a; Pidoux 1989; Laureys and De Vuyst 2014), which is why these large microbial groups were investigated throughout the sequencing and identification process using metabarcoding. At the species level, about 9 bacteria were identified (Table 4.4 and Figure 4.4), to which a name was assigned only if the similarity was >0.95. These species included *Lactobacillus ghanensis, Lactobacillus casei/paracasei, Lactobacillus harbinensis/perolens, Lactobacillus hilgardii, Lactobacillus diolivorans, Lactobacillus parabuchneri, Lactobacillus satsumensis, Oenococcus alcoholitolerans*, and *Oenococcus oeni*. In 2011, Gulitz *et al.,* reported the presence of 8 bacterial species. *(L. casei, L. hilgardii, L. hordei, L. nagelii, L. citreum, L. mesenteroides, A. fabarum, and A. orientalis).* On the other hand, in a more recent studies, Verce *et al.,* (2019) and Eckel *et al.,* (2020) reported different bacterial species, including *L. harbinensis, L. hilgardii, L. nagelii, L. paracasei,* and species like *L. hordei/mali, Bifidobacterium aquikefiri, Bifidobacterium tibiigranuli, Oenococcus oeni, Oenococcus kitahare. L. diolivorans, L. parabuchneri* and *L. satsumensis* are species that could be a distinguishing feature of Latin American grains, as they have not been widely reported for water kefir grains of European origin.

The functional properties inherent to the bacteria present in the WKGs are important for recognizing their role in the development of fermentations in various emerging foods. For example, in this preliminary study, for WKGCOL3 (see table 4.4), the most abundant lactic acid bacteria are *L. ghanensis, L. casei, L. harbinensis/perolens,* and *L. hilgardii*, with average relative abundances of 44.19%, 16.26%, 11.90%, and 9.92%, respectively. For *L. harbinensis/perolens*, it was found that the growth was favored at a temperature of 37°C on both substrates (panela and chancaca) and decreased at 21°C (for WKGCOL3), on the contrary (in the same grain), *L. hilgardii* shows an increase in its % abundance at 21°C. In the optimization study carried out in this research, a close relationship was observed between the increase in biomass and the treatments that considered temperatures between 20 and 23°C. This can be explained by the fact that *L. hilgardii*, an excellent producer of dextran, showed maximum growth at 21°C. It is worth noting that dextran is the compound that mainly constitutes the structure of the grains. On the other hand, the presence of *L. casei/paracasei* stands out as a microorganism recognized for its probiotic potential, which makes this fermented beverage a food with high added value.

Figure 4.3 shows the average relative abundance of the different bacterial genera in each kefir grain under the evaluated conditions. As shown in Table 4.4, for grain WKGCOL3, *Lactobacillus*

predominates with an average relative abundance of 96%, followed by *Acetobacter* with 1.8%, *Gluconobacter* with 1.0% and *Oenococcus* with 1.5%; and for WKGCH2, *Lactobacillus* with 96%, *Acetobacter* with 2.7%, *Gluconobacter* 1.1% and *Oenococcus* 0.5%, indicating that *Lactobacillus* largely dominates this fermentation process and therefore the lactic acid content is mainly produced by this microbial genus. Furthermore, it is confirmed that the high abundance of these microorganisms gives water kefir an important functional potential, as many of these bacteria are known for their GRAS status and probiotic potential. It is also important to note that during fermentation, lactic acid bacteria-BAL dominate and acetic acid bacteria- AAB are underrepresented, so lactic acid as the main metabolite contributes greatly to the flavor development of the fermented beverage and acetic acid could be found in very low percentages. The latter will be contrasted later in the optimization study.

The presence of *Oenococcus alcoholitolerans* and *Oenococcus oeni* is also noteworthy because the genus *Oenococcus* is important in malolactic fermentation, which is characterized by the conversion of L-malic acid to L-lactic acid and CO_2, leading to a reduction in acidity and having a generally positive and significant effect on organoleptic aspects. (Davis *et al.,* 1985; Krieger 1993; Liu 2002). This is mentioned because the control of the acidity level is important to prevent the grain death reported by (Laureys and De Vuyst, 2017b). Furthermore, this study aims to develop a standard beverage with high sensory quality for its potential commercialization.

The preliminary study aimed to evaluate a change in substrate and temperature to observe how these might affect the development of microbial communities and whether they were significantly representative of the main microbial groups (BAL, LEV and AAB) and species present. By observing the changes, one can infer whether the community remains stable or, on the contrary, whether these changes can lead to significant differences in the presence of populations. This was analyzed by calculating the standard deviations for each microbial genus, deviations related to both the overall mean abundance of each microbial group and the mean according to the independent variation of temperature or substrate. The Appendix 8.4, Table B shows the standard deviations calculated for both grains (WKGCOL3 y WKGCH2).

The microbiological composition in terms of genus and species is the same, and the relative abundance is also very similar for both grains. This allows us to conclude that these microbial consortia have a common origin and are simply adaptations to different substrates specific to the regions from which they were collected. The most significant differences can be found by comparing the microbiota of these grains with what has been reported by different authors, as we saw earlier, indicating that the substrate

can indeed induce significant changes in the microbial composition. However, it is perhaps the adaptation to the geographical conditions of the environment that leads to important evolutionary changes in the microbiology of the grains and in the abundance of the organisms present.

According to the standard deviations found by data group (see Table B in Appendix 8.4), it can be observed that the genera that showed significant changes (SD) in terms of general variation across all the experiments were *Lactobacillus*, followed by *Acetobacter* for both grains, and it was *Oenococcus* that showed the least dispersion with SDs less than 1. This indicates that each group of microorganisms is particularly affected by the different conditions applied to the fermentations, which may be due to their metabolic capabilities to consume certain substrates and the optimal temperature ranges at which they operate. For this reason, it is necessary to analyze the dispersion according to the specific variability regarding substrate or temperature. The standard deviations between the experiments with different substrates (S) but at the same temperature were much smaller or not significant compared to the experiments where different temperature conditions (°C) were evaluated but with the same substrate. The above indicates that different temperature states have significant effects on the relative abundance of the genus *Lactobacillus* in water kefir grains. On the other hand, for WKGCH2, less population variability was observed when different substrates were evaluated compared to when different temperatures were evaluated. However, in the case of the experiments where the temperature was maintained at 21°C but with different substrates, a significant change occurred in the populations of *Lactobacillus*. This may be due to the operating temperature fluctuating during the process due to a failure in the temperature control of the incubation equipment, or it may be due to the diversity of sensitivities that each species exhibits at 21°C, a case that will be analyzed further.

For the *Acetobacter* in relation to WKGCOL3 it was observed that when different substrtate were evaluated while keeping the temperature at 21°C, significant changes also occurred, although the variations in this genus were greater when different temperatures were evaluated. In the case of WKGCH2, it is also observed that the variability of *Acetobacter* is lower when evaluationg different substrates, where for both grains it was observed that the variability in abundance with respect to temperature was much more significant in fermentations with Chancaca. This indicates that the sensitivity of these microbial groups to different temperature states increases as a function of the substrate used. According to the Spanish Foundation for the Development of Animal Nutrition (FEDNA), it is known tht beet molasses (like chancaca) are rich in sodium and chlorine, while cane molasses (like panela) are rich in calcium and magnesium. This may indicate, as reported by Laureys and De Vuyst in 217b that the presence of calcium in panela allows this community to thrive and be more stable. In the case of *Gluconobacter*, the overall variation was less pronounced for both grains; however, the largest

variations (SDs greater than 1) were observed in experiments where different substrates were evaluated at 21 °C and in experiments where differents temperatures were evaluated in fermentations with chancaca this may indicate that there were also temperature control failures in the COL3-CH-21 and COL3-PAN-21 experiments and further supports the hypothesis that panela provides greater stability to the microbial community. Finally, in the case of *Oenococcus*, no significant variations (SD less than 1) in the population were found in all cases and for both grains. For WKGCH2, the greatest dispersion was also observed in the cases of fermentation with chancaca where different temperatures were evaluated, and for WKGCOL3, the greatest variability also occurred in the experiment where different substrates were evaluated and the temperature was maintained at 21 °C. Therefore, based on all the data analyzed for the experiments COL3-CH-21 and COL3-PAN-21, it is possible that there were failures in the temperature control of the equipment or that the interferences demonstrated using chancaca may have affected the dispersion of the data.

The same analysis was performed to understand the changes related to temperature and substrate, and it was observed that for both grains, greater changes occurred in the abundance of the *Lactobacillus* species (*L. ghanensis, L. harbinensis/perolens, L. hilgardii, L. casei/paracasei*, and *L. diolivorans*). It is worth noting that these species are the most abundant in the WKGs; therefore, they significantly influenced the variations and deviations observed with respect to temperature states in terms of standard deviation at the genus level. The species affected by the evaluation of different substrates were all those mentioned above, with the exception of *L. diolivorans*.

This may indicate that these *Lactobacillus* species are particularly sensitive to different temperature conditions and also respond to substrate variations, especially when exposed to temperatures outside their optimal range, as was the case in the experiments conducted at 21°C. According to all the previous analyses, it can be stated that the different temperatures evaluated in the different fermentations represent moderate changes in the microbial abundance populations, when considered from the perspective of genus analysis. This perspective is useful to observe the behavior of the WKG in a more practical way. In addition, it is necessary to approach the operation of this type of biofeeding process with a more macro perspective that allows to optimize and operate processes of this nature in real environments.

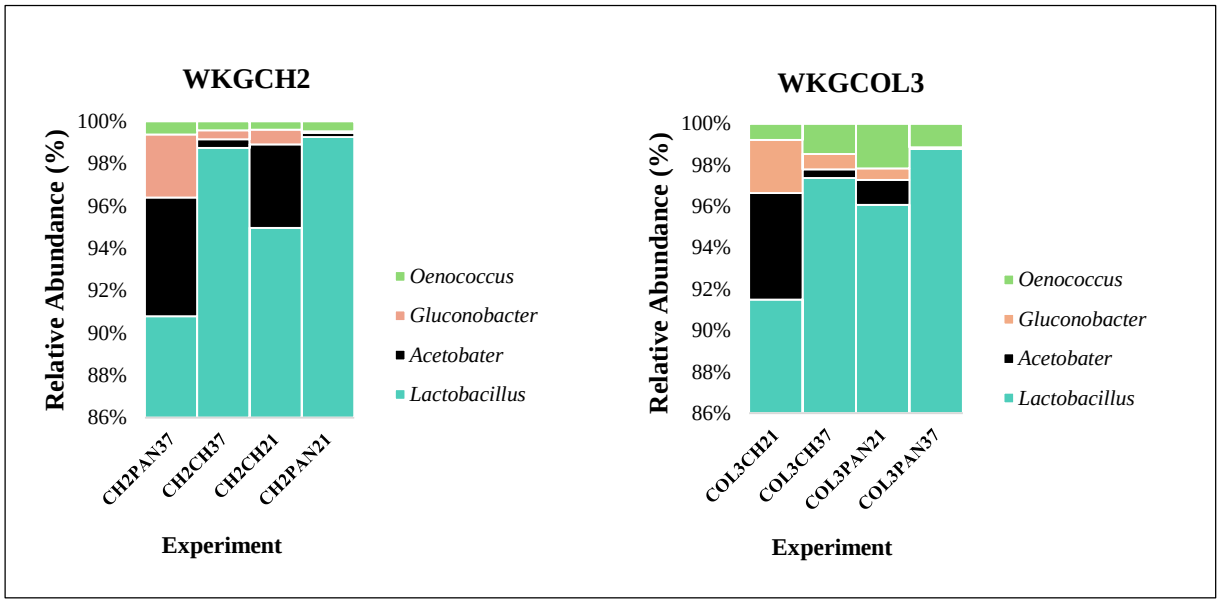

Figure 4.3. *Relative abundance (%) of bacterial genera present in both grains for preliminary experiments. At the species level, for both grains the same microorganisms predominate. COL3(Colombian water kefir grain), CH2(Chilean water kefir grain),21 (temperature at 21°C), 37(temperature at 37°C). CH (Chancaca), PAN (Panela). See methodology section 2.10.*

As can be seen in Figure 4.3, the relative abundance of both grains is very similar in terms of composition and microbial proportions, which may be due to the great stability at the species level that this community has on the Latin American continent. This was already discussed in the analysis of the SDs in the experiments subjected to substrate and temperature changes, and it was found that the community at the level of large microbial groups was not significantly modified by the substrate change and that it only responded to temperature changes in some species of *Lactobacillus.* Comparing the two relative abundance plots, we can see that the pattern or profile of the genus proportions is very similar. This further indicates that the community is stable, because although the grains are adapted to different countries and substrates, it is quite interesting that their microbiological compositions are the same, with very minimal variations in terms of abundance.

Table 4.4. *The percentages of relative abundance for each grain and operating condition under evaluation of the various bacterial species and genera found in the preliminary study using metabarcoding analysis.*

Relative Abundance (%) WKGCOL3							
Species	***COL3CH 21***	***COL3CH 37***	***COL3PAN 21***	***COL3PAN 37***	***Average Relative Abundance (%)***	***Genus***	***Average Relative Abundance (%)***
Lactobacillus ghanensis	48.56	40.88	40.24	47.07	44.19		
Lactobacillus_casei/ paracasei	14.43	16.91	20.53	13.18	16.26		
Lactobacillus_ harbinensis/perolens	6.76	15.15	10.50	15.20	11.90		
Lactobacillus hilgardii	14.76	8.73	11.03	5.16	9.92	*Lactobacillus*	95,59
Lactobacillus diolivorans	4.28	10.39	5.10	11.47	7.81		
Lactobacillus parabuchneri	2.66	3.54	4.66	4.27	3.78		
Lactobacillus satsumensis	0.03	0.00	0.01	0.01	0.01		
Lactobacillus_perolens	0.03	0.01	0.03	0.01	0.02	*Acetobacter*	1,84
Acetobacter (various species)	5.46	0.47	1.26	0.03	1.80		
Gluconobacter (various species)	2.72	0.82	0.58	0.05	1.04	*Gluconobacter*	1,06
Oenococcus alcoholitolerans	0.57	0.40	1.78	0.53	0.82		
Oenococcus oeni	0.27	1.21	0.45	0.73	0.67	*Oenococcus*	1,51
Relative Abundance (%) WKGCH2							
Species	***CH2CH 21***	***CH2CH 37***	***CH2PAN 21***	***CH2PAN 37***	***Average Relative Abundance (%)***	***Genus***	***Average Relative Abundance (%)***
Lactobacillus ghanensis	30.47	35.48	41.23	43.36	37.63		
Lactobacillus casei/ paracasei	18.97	14.73	19.40	18.42	17.88		
Lactobacillus harbinensis/perolens	19.02	24.54	14.59	15.25	18.35		95.73
Lactobacillus hilgardii	10.43	13.08	8.08	8.10	9.92	*Lactobacillus*	
Lactobacillus diolivorans	3.77	5.13	3.46	5.88	4.56		
Lactobacillus parabuchneri	5.15	4.62	3.58	3.52	4.22		
Lactobacillus satsumensis	2.02	0.56	2.62	1.63	1.71		
Lactobacillus_perolens	0.62	0.52	0.41	0.37	0.48	*Acetobacter*	2.67
Acetobacter (various species)	5.82	0.43	4.08	0.21	2.64		
Gluconobacter (various species)	3.08	0.46	0.72	0.09	1.09		
Oenococcus alcoholitolerans	0.63	0.40	0.40	0.47	0.47	*Gluconobacter*	1.10
Oenococcus oeni	0.02	0.05	0.01	0.02	0.02	*Oenococcus*	0.50

Figure 4.4 below shows the distribution of the species identified for both grains. The average relative abundance of each species in all treatments. *L. ghanensi*s was the most predominant species with a maximum of 44% for WKGCOL3 and 38% for WKGCH2. For the grain from Colombia, the most represented species were *Lactobacillus ghanensis*, *Lactobacillus casei / paracasei, Lactobacillus harbinensis / perolens* and *Lactobacillus hilgardii* with 44, 16, 12 and 10% respectively, while for the Chilean grain *Lactobacillus ghanensis, Lactobacillus_casei / paracasei, Lactobacillus harbinensis / perolens* and *Lactobacillus hilgardii* were found to have average relative abundances of 38, 17.8, 18 and 10% respectively, which is very similar if a comparison is made for both grains, this may indicate that both water kefir share the same lineage. In Figure 4.4 is detailed, the species distribution pattern is very similar in both microbial communities.

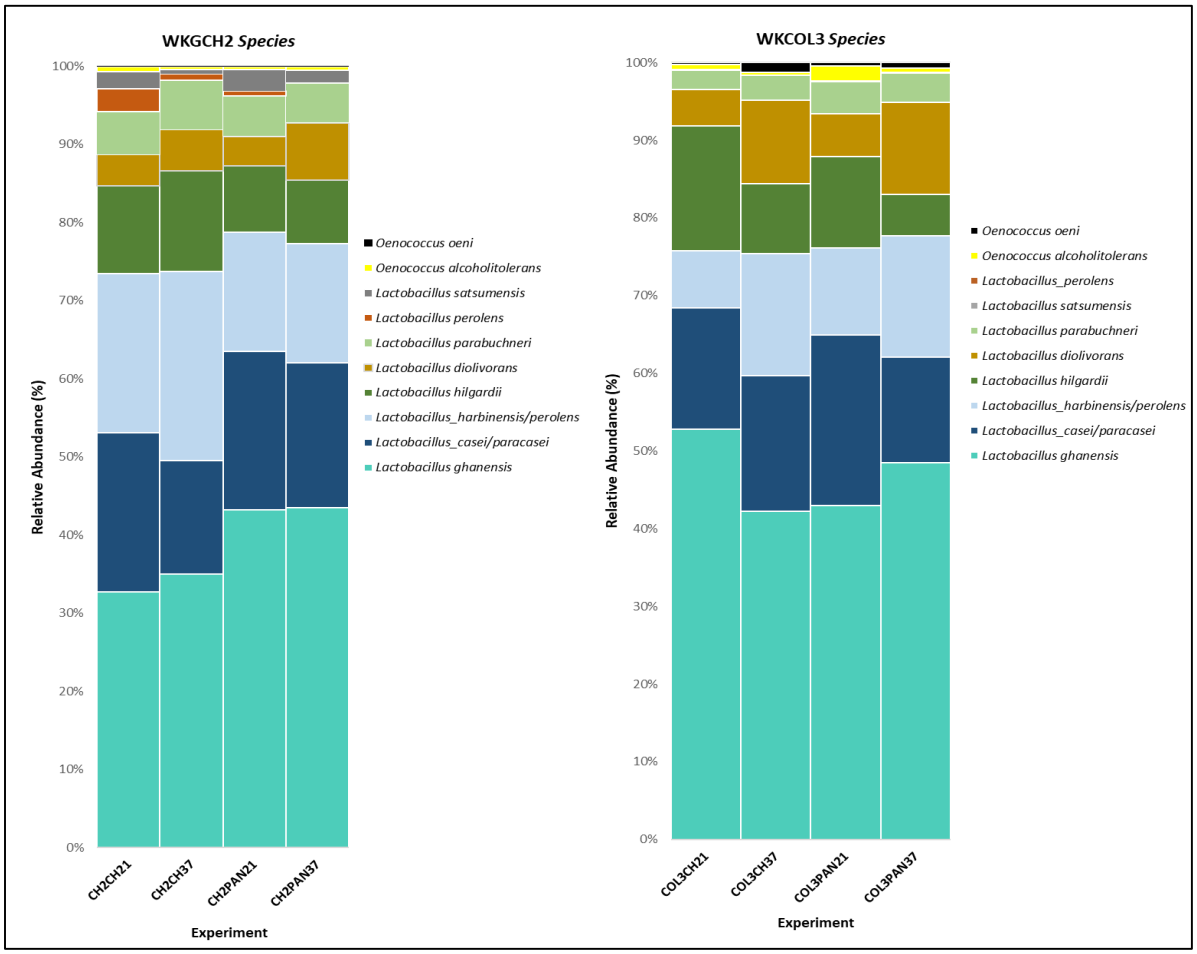

Figure 4.4. *Relative abundance (%) graphs of bacterial species present in both grains for preliminary experiments conducted with two different substrates (panela and chancaca) and at two different temperatures (21 and 37°C). COL3 (Colombian water kefir grain), CH2 (Chilean water kefir grain),21 (temperature at 21°C), 37(temperature at 37°C); CH (Chancaca), PAN (Panela). See methodology section 2.10.*

Gulitz in 2011 (Anna Gulitz *et al.,* 2011a) reported that the composition of the microbiota varied between grains, with *Lactobacillus* species representing approximately 25.2% and *Acetobacteria* representing 4.7%. Comparing this study with the previously mentioned gender data in WKGCOL3 and WKGCH2, the percentage difference between the grains evaluated by Gulitz *et al.,* (2011) and those in this study is 70.8% for *Lactobacillus* and 2.2% for *Acetobacter*. In the case of the increase in *Lactobacillus* in this study, the abundance was higher; the opposite was true for *Acetobacter*. The findings of Gulitz *et al.,* 2011, regarding the decrease of *Acetobacter* can be inferred because of the use of anaerobic fermentation, since it is known that AAB are obligatory aerobes, and oxygen becomes a real limiting factor for their growth. However, some AAB can grow even though the anaerobic conditions present during alcoholic fermentation are not favorable for their development (Guillamón and Mas 2011; Mamlouk and Gullo 2013). This could explain the presence of *Acetobacter* due to the significant abundance of yeasts in the fermentations of grains WKGCOL3 and WKGCH2, since anaerobic fermentations were implemented in the methodology, and this condition could allow *Acetobacter* to increase in number and, consequently, modify the physicochemical characteristics of the fermented products and the sensory perception. In this preliminary study, the *Acetobacter* could not be identified at the species level, maybe because *acetobacteria* were very poorly represented in the fermentation process; some studies managed to identify non-lactic bacteria (non-LABs), such as *Acetobacter fabarum* and *Acetobacter orientalis* (Gulitz *et al.,* 2011). Fiorda, in 2017, was the first to identify *Acetobacter lovaniensis* and *Kazachstania* aerobia in water kefir. There are also studies on Brazilian kefir (Miguel *et al.,* 2011), which first reported the presence of acetic acid bacteria species such as *Gluconobacter liquefaciens* in water kefir grains, and finally Sarikkha in 2015 (Sarikkha *et al.,* 2015) identified *Gluconobacter japonicus*.

The *Lactobacilli* and yeasts in water kefir predominate and promote the growth of each species in the symbiotic interaction. A 2013 study investigated how *Z. florentina* and *S. cerevisiae* can mutually provide essential amino acids to *L. nagelii* and vitamin B6 to *L. hordei.* The release of essential nutrients from the yeasts to the *lactobacilli* is self-induced by the *lactobacilli*, as only they are stimulated by the co-culture. This interaction is based on the adjustment of the physicochemical environment through the production of organic acids by *lactobacilli* and the consequent acidification of the medium. Given this, the balance of the microbial community of species within the grain depends on the cultivation variables and the species that inhabit it (Stadie *et al.,* 2013). It is worth noting that unrefined sugar has a composition that, according to the Food and Agriculture Organization of the United Nations (FAO), is more than just a sweetener (Jaffé 2015); it is considered a food product with high nutritional value, making it the ideal substrate for this type of fermentation with WKG. In the case of this study, *L. hilgardii* makes up 5.3% of WKGCOL3 and 10% of WKGCH2. As mentioned earlier, the species proportions

vary between grains from different regions. In this study, the abundance of *L. hilgardii* is higher than that reported in grains of German origin. (Gulitz *et al.,* 2011). In the stage 4 of this study were presented comparisons of biomass increase among three different grains (the two selected grains -WKGCOL3 and WKGCH2- and a grain from Germany), which may support the correlation between *L. hilgardii* and dextran production (the main component of water kefir grains).

The presence of *Oenococcus* in Belgian water kefir grains has been previously reported in metagenomic analyses with abundances up to 11.0% in Belgian WKG, with species such as *Oenococcus alcoholitolerans* and *Oenococcus oeni* (Verce, De Vuyst, and Weckx 2019; Minervini *et al.,* 2019). The discovery of this genus and these species is a novel finding that redefines the microbiological composition of WKG, which is currently described as a symbiotic association between LAB, AAB, and yeasts (Horisberger 1969; Pidoux 1989; Ward Marshall 1892; David Laureys and De Vuyst 2014b; Anna Gulitz *et al.,* 2011b). This definition is still used in current research, but due to advances in genomic sciences, it needs to be reconstructed as *Oenococcus* is classified in different ways.

4.4. Preliminary metabarcoding study of water kefir grains. (Yeast composition)

Table 4.5 and Figure 4.5 show the relative abundance of the identified fungal genera. In WKGCOL3, the genus *Dekkera* predominates at 46%, followed by Saccharomyces at 28% and *Zygotorulaspora* at 22%. With smaller representation are *Saccharomycodes* in 2.6%, *Schizosaccharomyces* in 1.52% and *Candida* in 0.64%. In WKGCH2*, Zygotorulaspora* predominates in 56%, *Dekkera* in 27%, *Saccharomyces* in 16%, *Saccharomycodes* in 0.16% and *Candida* in 0.36%. The difference between the two grains was that *Schizosaccharomyces* pombe was not detected in the Chilean kefir grains. According to the above, the same phenomenon is observed with bacteria, since the genera and species of yeast involved in both grains are the same. This, in turn, reinforces the hypothesis of a common adapted grain that preceded all the existing ones and that arrived or evolved in Latin America. As in the analysis carried out for bacteria, the standard deviations (SD) of the treatments were calculated according to the changes in temperature (°C) and substrate (S), with the aim of validating whether the system was disturbed and how stable the microbial community is in terms of relative abundance and the presence or absence of certain species when intervening in the biological system.

Table 4.5. *The percentages of relative abundance for each grain and operating condition under evaluation of the various bacterial species and genera found in the preliminary study using metabarcoding analysis.*

WKGCOL3 microorganism species	Relative Abundance (%)				Average Relative Abundance (%)	WKGCOL3 microorganism genus	Average Relative Abundance (%
	COL3CH 21	COL3CH 37	COL3PAN 21	COL3PAN 37			
Zygotorulaspora florentina	31,38	1,94	39,49	0,77	18,40	*Zygotorulaspora*	21,75
Schizosaccharomyces pombe	1,02	1,96	1,49	2,16	1,66	*Dekkera*	46,02
Saccharomycodes ludwigii	3,33	1,87	3,11	1,29	2,40	*Saccharomycodes*	2.62
Saccharomyces cerevisiae	20,82	35,05	32,56	26,84	28,82	*Saccharomyces*	27.45
Dekkera bruxellensis	42,03	59,13	23,02	68,93	48,28	*Schizosaccharomyces*	1,52
Candida californica	1,43	0,05	0,33	0,00	0,45	*Candida*	0,64

WKGCH2 microorganism species	Relative Abundance (%)				Average Relative Abundance (%)	WKGCH2 microorganism genus	Average Relative Abundance (%
	CH2CH 21	CH2CH 37	CH2PAN 21	CH2PAN 37			
Zygotorulaspora florentina	83,83	8,99	72,51	28,74	48,52	*Zygotorulaspora*	55,95
Dekkera bruxellensis	9,70	66,99	18,47	31,61	31,69	*Dekkera*	27,27
Saccharomycodes ludwigii	5,76	23,79	8,47	39,23	19,31	*Saccharomyces*	16,26
Saccharomyces cerevisiae	0,08	0,17	0,10	0,38	0,18	*Saccharomycodes*	0,16
Candida californica	0,63	0,06	0,45	0,04	0,29	*Candida*	0,36

Based on the data dispersion analysis, the genera and species (since only one species per genus could be identified) showing the highest general level of variability in both grains are *Zygotorulaspora*, *Saccharomyces,* and *Dekkera.* The greatest variation was observed for *Zygotorulaspora*, with a SD of 35.48 (See Appendix 8.4, Table C) in the case of WKGCH2 and 19.96 in the case of WKGCOL3. According to the dispersion analysis between the experiments carried out only with the evaluation of different temperatures or only with the evaluation of different substrates, it was found that the same genera presented significant variations, highlighting that the greatest changes occurred in the experiments where different temperatures were evaluated keeping the substrate constant. The same was true for the bacterial genus groups. In conclusion, yeasts, especially *Zygotorulaspora* and *Dekkera*, are more sensitive to different temperature states than to substrate states. Among the yeasts that showed

variability, the one that changed the least in abundance between treatments was Saccharomyces for both grains, showing its great adaptive capacity and its technological potential as one of the best-known yeasts in the industry. It should be noted that Schizosaccharomyces did not show significant variations in any case, but it is much underrepresented, so it is very likely that the abundance was very similar at the beginning and at the end of the fermentation, since it is not present in large numbers, the possibilities of colonization and access to substrate are lower. Although all genera showed variability according to the different stages applied, the experiments in which different substrates were evaluated and a temperature of 21 °C was maintained showed the greatest stability according to the SD analysis

The arithmetic means of the above-mentioned process, with smaller variations (constant temperature of 21 °C), showed that the strains had a higher relative frequency in both grains, so that we can say that the strains present in these grains have a high technological potential. The brewing industry, for example, is trying to adapt these microorganisms to lower temperature conditions since yeasts fermenting below 20 °C can have slower metabolic rates and higher catalytic efficiencies than mesophylls, making them much more interesting for biotechnological applications (Liszkowska *et al.,* 2021).

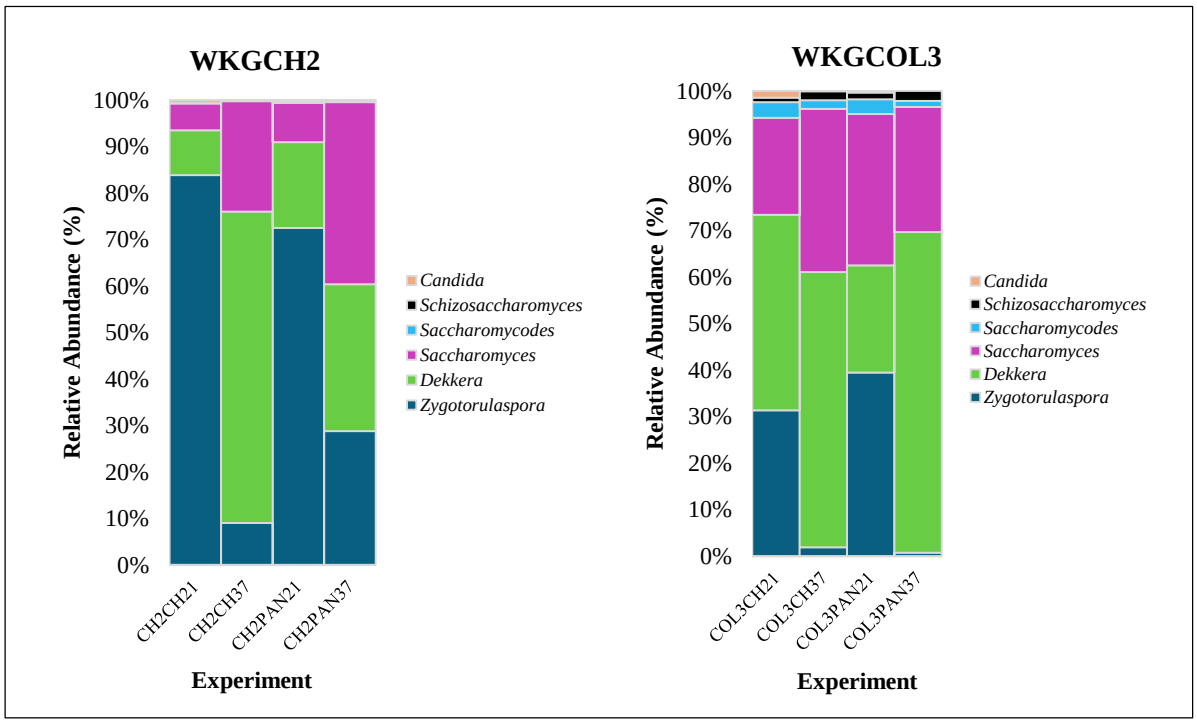

Figure 4.5. *Plots of relative abundance of genera (%) in both grains (WKGCOL3 and WKGCH2) for the preliminary metagenomic study. COL3 (Colombian water kefir grain), CH2 (Chilean water kefir grain), 21 (temperature at 21°C), 37 (temperature at 37°C), CH (Chancaca), PAN (Panela). See Section of Methodology.*

Saccharomyces cerevisiae is the most abundant yeast in WKG of different origins and is therefore considered to be a key member of the microbial community (Verce, De Vuyst, and Weckx 2019; David Laureys *et al.,* 2018; D. Laureys and De Vuyst 2017a; Zanirati *et al.,* 2015; Marsh *et al.,* 2013; A. Gulitz *et al.,* 2013; Galli *et al.,* 1995). *Zygotorulaspora florentina* is also considered the predominant microorganism in most studies (Lynch *et al.,* 2021b; A. Gulitz *et al.,* 2013; Anna Gulitz *et al.,* 2011a; Magalhães *et al.,* 2010); (Stadie *et al.,* 2013; Anna Gulitz *et al.,* 2011b). Since *S. cerevisiae* can convert sucrose to the monosaccharide's fructose and glucose (Ikram-Ul-Haq and Ali 2007), the latter are readily metabolized by the other yeasts, possibly through alternative metabolic pathways. (Kurtzman and Robnett 2003). This may be a response to the presence of other yeast species, indicating that Latin American grains may be more diverse than those originating in Europe. For example, Belgian water kefir is typically dominated by only two species, *S. cerevisiae* and *D. bruxellensis* (Verce, De Vuyst, and Weckx 2019; David Laureys and De Vuyst 2014b). The yeast species and/or genera found in this study have also been identified by other authors, such as *Dekkera bruxelensis* (Marsh *et al.,* 2013; David Laureys *et al.,* 2018) and *Candida californica* (Martinez-Torres *et al.,* 2017). With respect to yeasts of the *Saccharomycodes* and *Schizosaccharomyces* genus, no reports of their presence in these types of microbial communities were found, which may represent a novelty in this field of study. Yeasts are a relatively diverse group in the microbial communities evaluated in this study, which could be a precursor of different aromas and flavors in the resulting fermented beverages.

Figures 4.5 and 4.6 show the distribution of the relative abundances of yeasts for both grains, revealing significant differences with changes in substrate and temperature; it can also be observed that, comparing the treatments carried out at 21°C, for the case of WKGCH2, the distribution of both genus and species is very similar. This allows us to conclude that a condition that confers stability on the fungal species of this grain is closely related to this temperature. In the case of WKGCOL3, the temperature of 37 °C presented better stability according to its SD, indicating that the yeasts present in this grain behave better at mesophilic temperatures, which are typical of Colombia. It can also be observed that the microorganisms that give greater variability are *Dekkera, Zygotorulaspora*, and *Saccharomyces,* which may indicate that they are modulated by competition for substrate. In the case of WKGCOL3, *Dekkera* and *Saccharomyces* behave stably at 37°C, almost completely displacing the growth of *Zygotorulaspora*, which grows better in the same grain at 21°C and predominates in almost equal proportions to *Dekkera* and *Saccharomyces.* In the case of WKGCH2, the yeast *Zygotorulaspora* behaves stably at 21°C, but its abundance decreases significantly at 37°C without disappearing; therefore, it is evident that its growth is better at lower temperatures more typical of the country of Chile and that its presence is a marker of grains adapted to this country and to the chancaca substrate.

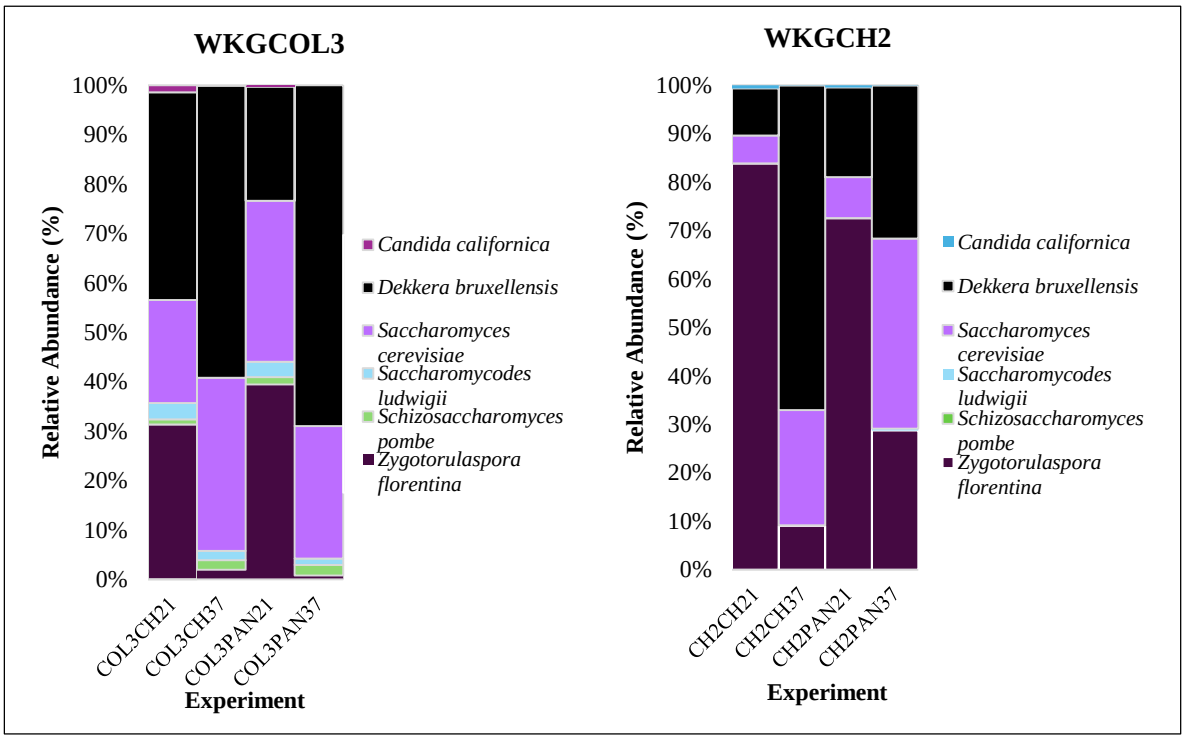

Figure 4.6. *Species relative abundance (%) in both grains. See Table 3.4 above, where the conditions of each experiment are explained. COL3 (Colombian water kefir grain), CH2 (Chilean water kefir grain), 21 (temperature at 21°C), 37(temperature at 37°C); CH (Chancaca), PAN (Panela). See methodology section 2.10.*

4.5. Determination of optimal operating conditions for water kefir grain fermentations by Response Surface Methodology (experiment at 500 ml)

The experimental data for Colombian grains (WKGCOL3) and Chilean grains (WKGCH2) are presented in Tables 4.6 and Table 4.7, respectively. For the fermentations produced with WKGCOL3, the final grain (FG) contents varied between 98 and 150 g/L, lactic acid (LA) had contents between 1.3 and 9.3 g/L, acetic acid (AA) varied between 0.4 and 0.9 g/L, ethanol (EtOH) between 7 and 25.5 g/L, and for the sugars, glucose (GLUC) had contents between 2.15 and 11.65 g/L, fructose (FRUC) between 3.90 and 11.45 g/L, and finally sucrose (SUCR) between 6.35 and 61.9 g/L, while those fermented with WKGCH2 had FG contents between 91 and 123 g/L, LA between 1.4 and 10.75 g/L, AA between 0.15 and 0.70 g/L, EtOH between 4.15 and 21 g/L, GLUC with values between 4.15 and 12.80 g/L, FRUC between 8.75 and 16.50 g/L, and finally SUCR between 11 and 48.55 g/L.

According to these restrictions and obtaining fermented products with the desired compositional characteristics in terms of LA, AA, EtOH and sugars, it was found that in the case of LA in both grains,

the contents were above 1.3 g/L. However, under operating conditions at temperatures above 37°C, the content of this acid rises to around 10 g/L, indicating that under these conditions LABs are more active and therefore their metabolism is favored. As already mentioned, this group of microorganisms mainly produces lactic acid, and their optimal growth temperature is between 30 and 40 °C (Slizewska et al, 2020).

As expected, the AA content was very low in both grains, with values below 0.01% of the total content, so it can be stated that it is possible to achieve fermentations with very low AA content under all conditions within the operational range of the evaluated samples. This result is expected because an anaerobic atmosphere is unfavorable for AAB, whose metabolism and growth are affected by the absence of oxygen (Guillamón and Mas 2011; Anna Gulitz *et al.*, 2011a), since acetate production occurs through an oxidative pathway of sucrose and ethanol fermentation. (Patel *et al.*, 2022; David Laureys *et al.*, 2016). It is important to note that AAB are obligately aerobic (Mamlouk and Gullo 2013). EtOH showed acceptable values under all conditions evaluated at 30°C for both grains, with acceptable average values considered to be between 0.7 and 1.5%. The operating conditions at 37°C showed ethanol contents above 1.5% in both grains. Alcohol content is of particular concern because food regulations can change depending on the target audience, product classification, and functionality. Therefore, if the sample space of this design does not account for the desired ethanol content in this product, the operating conditions would not be viable for the development of this product.

Sugar consumption was reduced in Condition 8 for both grains because the operating temperature (20°C) is outside the optimal temperature range for LAB and yeasts, which are the microbial groups most involved in consuming the sugars present. In the case of FRUC, this sugar showed lower uptake in both grains at the temperature of 40°C and the highest uptake at temperatures below 37°C, which may indicate that the range of activity of fructokinase involved in fructose metabolism is between 30 and 37°C, as reported by Macdonald et al, 2005. The latter may be the reason why FRUC consumption occurs under different conditions than SUCR and GLUC, which at 20°C had the lowest consumption.

It is desirable to operate under conditions that allow the grains to grow sufficiently to maintain a viable and active starter culture over time. In all experiments, it was observed that both grains maintained their viability based on their easy activation for biological replication, pH decrease, and formation of acids and ethanol. In addition, grains larger than 0.5 mm were observed, showing an elastic and translucent structure, indicating that the grains were in good condition, as reported by Laureys *et al.*, (2016). It can be observed that in both grains, greater growth occurred at 30°C and when the agitation was very low, not exceeding 80 rpm. This is important because violent mechanical movement apparently causes growth to stop, while slow agitation may improve substrate turnover and consumption. At this scale (experiments

carried out at 500 mL), and later, when comparing the results at a global level with the experiments carried out at 250 mL, the above can be confirmed, i.e., violet agitation in a larger space can interfere with fermentation and cause substrate exchange to be inconsistent and heterogeneous since the grains can break and their irregular shape makes the results of product formation very variable; therefore, standardization is much more complex at high agitation.

4.5.1. Evaluation of the effect of operating conditions using Response Surface Methodology

The statistical evaluations are shown in Appendix 8.5, Table B, and the fitted regression equations are shown in Table 4.8 for both grains. For the Colombian grain, the responses that can be adequately fitted with a second-order equation are LA and FG. For the Chilean grain, the response variables LA and FRUC showed appropriate fits with a second-order equation (Due to the fact that there were only two response variables with good fits, and to validate the agitation issue, it was necessary to run a second experiment at 250 mL). For the evaluation of the equation, the coefficients R^2, adjusted R^2 (R^2adj), adequate precision, and coefficient of variation (CV) were calculated. The adequate precision (signal-to-noise ratio comparing the predicted values with the mean prediction error) was greater than 4, indicating that the equations for the mentioned responses can be used for predicting effects and behavioral effects. The trends in product formation and biomass and substrate consumption are very similar for both grains, indicating that the significant similarities indicate the presence of microbial groups and common species in similar relative abundances.

Table 4.6 *Measured data for the response parameters in the fermentation of WKGCOL3, final grain (FG), lactic acid (LA), acetic acid (AA), ethanol (EtOH), glucose (GLUC), fructose (FRUC), and sucrose (SUCR), respectively. Data reported are in g/L.*

Run	Agitation RPM (X_1)	Temperature °C (X_2)	Exp. Code	FG (Y_1)	LA (Y_2)	AA (Y_3)	EtOH (Y_4)	GLUC (Y_5)	FRUC (Y_6)	SUCR (Y_7)
				(g/L)						
1	136.57	22.93	COL3EXP1	115.57	3.25	0.85	16.50	5.70	9.20	24.50
2	160.00	30.00	COL32EXP2	136.64.	3.30	0.70	9.00	8.75	7.00	45.10
3	136.57	37.07	COL3EXP3	99.34	9.30	0.95	25.50	2.15	6.25	6.35
4	80.00	40.00	COL3EXP4	97.78	7.55	0.45	16.00	7.25	11.45	22.35
5	23.43	37.07	COL3EXP5	110.89	6.25	0.75	15.50	7.75	4.85	38.15
6	0.00	30.00	COL3EXP6	130.81	4.05	0.75	9.00	9.00	7.55	48.05
7	23.43	22.93	COL3EXP7	150.32	3.45	0.90	17.00	2.85	3.90	17.20
8	80.00	20.00	COL3EXP8	109.95	1.30	0.40	7.00	11.65	7.80	61.90
9	80.00	30.00	COL3EXP13	113.76	2.90	0.50	13.00	9.20	5.80	48.50
10	80.00	30.00	COL3EXP13.2	113.51	3.20	0.60	12.00	7.40	5.40	39.10
11	80.00	30.00	COL3EXP13.3	116.74	3.20	0.70	13.00	7.70	5.70	40.60
12	80.00	30.00	COL3EXP13.4	115.81	3.10	0.60	13.00	7.65	5.45	40.05
13	80.00	30.00	COL3EXP13.5	114.20	3.30	0.55	12.70	7.85	5.75	45.05
14	80.00	30.00	COL3EXP13.6	116.05	3.00	0.65	13.02	7.60	5.60	43.63

Table 4.7. *Measured data for the response parameters in the fermentation of WKGCH2, final grain (FG), lactic acid (LA), acetic acid (AA), ethanol (EtOH), glucose (GLUC), fructose (FRUC), and sucrose (SUCR), respectively. Data reported are in g/L.*

Run	Agitation RPM (X_1)	Temperature °C (X_2)	Exp. Code	FG (Y_1)	LA (Y_2)	AA (Y_3)	EtOH (Y_4)	GLUC (Y_5)	FRUC (Y_6)	SUCR (Y_7)
							(g/L)			
1	136.57	22.93	CH2EXP1	98.10	3.50	0.50	14.00	8.00	11.85	33.10
2	160.00	30.00	CH2EXP2	120.89	4.45	0.30	7.50	7.75	8.75	39.15
3	136.57	37.07	CH2EXP3	91.47	10.75	0.70	21.00	4.60	11.00	11.35
4	80.00	40.00	CH2EXP4	92.13	6.90	0.25	14.50	6.85	16.50	11.00
5	23.43	37.07	CH2EXP5	96.49	8.75	0.40	16.00	6.70	10.00	25.70
6	0.00	30.00	CH2EXP6	117.01	5.80	0.35	7.50	9.10	13.10	35.40
7	23.43	22.93	CH2EXP7	123.21	4.55	0.50	16.00	4.15	10.75	15.40
8	80.00	20.00	CH2EXP8	96.55	1.40	0.15	7.00	12.80	13.10	48.55
9	80.00	30.00	CH2EXP13	96.13	4.10	0.50	15.00	8.00	10.00	31.30
10	80.00	30.00	CH2EXP13.2	95.23	3.80	0.40	14.00	8.30	9.10	35.20
11	80.00	30.00	CH2EXP13.3	95.00	3.80	0.50	13.00	8.00	9.70	31.90
12	80.00	30.00	CH2EXP13.4	121.10	3.90	0.45	14.50	8.56	9.98	33.56
13	80.00	30.00	CH2EXP13.5	123.21	3.75	0.53	14.90	8.38	9.90	31.98
14	80.00	30.00	CH2EXP13.6	108.43	3.95	0.56	13.90	9.00	10.01	34.00

The formation of biomass is slightly higher in all experiments for WKGCOL3, which is confirmed by the relative abundance of *L. hilgardii*, since this microorganism is more represented in WKGCOL3 than in WKGCH2 and is directly responsible for the formation of dextran in this type of grain. It may also be that the metabolic state of WKGCOL3 is more favored because fermentation occurs in its conventional substrate (cane NCS), unlike Chilean grain WKGCH2, whose conventional substrate is beet NCS. In the case of acetic acid, the formation occurred in greater quantities during the WKGCOL3 fermentation, which is confirmed by the percentage of abundance of AAB which, according to the metabarcoding, are more abundant in WKGCOL3 compared to WKGCH2.

4.5.2. Final Grain growth (FG) of water kefir fermentations

According to the results found in Table B ANOVA (see Appendix 8.5), the fitted equation for grain WKGCH2 was quadratic with a p-value of 0.3259. This indicates that all factors are significant for the equation. The correlation coefficient R^2 was 0.4631, the CV coefficient was 11.39%, and the adequate precision was 4.5805; these last two criteria also indicate that the FG variable is represented by the polynomial. For the WKGCOL3 model, the fit to the data set was also quadratic with a p-value <0.0179, indicating a good model fit. The R^2 was 0.7726, consistent with goodness of fit. The CV was 8,41% and the adequate precision accuracy was 8.984, which theoretically indicates that the model is reliable. The fitted equation indicates that all terms are significant.

The FG from WKG in Chile and Colombia that were subjected to temperature and agitation variations ranged from 91.47 to 123.21 g/L (treatments 3 and 7) and 97.78 to 150.32 g/L (treatments 3, 4, and 7) respectively, as shown in Tables 4.6 and 4.7. This indicates that the minimally agitated fermentation treatment (23.43 rpm) and temperature (22.93 °C) produced the highest grain growth in both cases, increasing the biomass in the WKGCOL3 case from 80 g/L to 150.32 g/L (an increase of 88 %) and in the WKGCH2 case from 80 g/L to 123.21 g/L (an increase of 54 %). This is in line with the optimization study for metabarcoding analysis, which found that for both grains, 22.93 °C and 137 rpm of stirring speed is the optimal temperature for increasing the abundance of lactic acid bacteria. It is very likely that the *Lactobacillus* species involved in the reproduction of the grain are mesophilic. In comparison with the final biomass values of water kefir grains reported by Laureys *et al.,* (2017), where a treatment of 96 hours at 21 °C showed an increase in grains of 56 %; a relationship is observed between the increase in biomass of grains at temperatures between 21 - 23 °C according to what this author published and what was found in this study.

It is known that *Lactobacillus* is responsible for the production of dextran, it has been reported that the optimal temperatures for dextran production are between 23°C and 30°C (Slađana *et al.,* 2014). Therefore, it is possible that the temperature assessed in condition 7 (23 °C) plays an important role in the increase of biomass. The FG variable showed a quadratic effect, which can be observed in the response surface plots, Figures 4.10a and 4.11a, where the amount of biomass is greater at lower temperatures. According to the results for both grains, comparing the maximum and minimum contents of FG (see tables 4.6 and 4.7) and the treatments under which they were obtained, it can be observed that there are similar behavioral trends for both grains, which helps to conclude that the models found for this variable can be extrapolated to other processes with FG.

4.5.3. Lactic Acid (LA) formation in WKG fermentation

Tables 4.6 and 4.7 show the LA values obtained for the grains WHGCOL3 and WKGCH2, respectively, which ranged from 1.30 to 9.30 g/L for WKGCOL3, and for WKGCH2, it was found that the formation of LA varied between 1.40 and 11 g/L. It can be observed that at lower temperatures, the amounts of this metabolite are lower. It also shows that there is a very similar trend in the formation of LA for both grains in all experiments, indicating that the models found can be used for other processes under similar conditions to WKG. For both grains, the lowest and highest amounts of LA correspond to experiments 8 and 3 respectively, where the highest amount of LA was present at a temperature of 37°C and a stirring

speed of 137 rpm. The effect of temperature and stirring on the formation of LA is of a quadratic order, as can be seen in Figures 4.10b and 4.11b.

The formation of lactic acid in this fermentation represents one of the most important metabolites due to the presence of lactic acid bacteria as a major microbial group of WKG. (Gulitz *et al.,* 2013). Laureys *et al.,* in 2017 reported values between 0.74 and 1.48 g/L of this compound for an aerobic process carried out over 72 hours at 21°C, which may indicate that aerobic conditions and low temperatures do not favor lactic acid production. This could be because lactic acid bacteria have a predominantly anaerobic metabolism (Smetanková *et al.,* 2012). In this study, as mentioned, the highest levels of LA were found in experiment 3 at the optimal growth temperature of LAB (37°C), confirming that this compound is abundant under anaerobiosis and at this temperature. According to what has been presented for the FG variable, at one of the lowest temperatures evaluated (23°C), microorganisms can enhance their metabolism towards the production of dextran and new cells, while at higher temperatures (37°C), microorganisms favor the formation of LA.

According to the results presented in Table B ANOVA (see Appendix 8.5), the fitted equation for grain WKGCOL3 was of a non-linear type. The factors and interactions showed significant effects in the regression; the coefficient of determination R^2 and the CV indicate that the equation can be used and is reproducible according to the criteria of adequate precision and R^2. For WKGCH2, a non-linear model was fitted with a p-value <0.0012; the R^2 indicates that the equation explains the relationship between the factors and the responses.

4.5.4. Ethanol (EtOH)) formation in water kefir fermentation

The ethanol models found in the WKGCH2 and WKGCOL3 fermentations, with values of p = 0.5263 and p = 0.4364, respectively, were quadratic, and the correlation coefficients R^2 were less than 0.4, indicating no evidence of a close relationship between the response variable and the factors. The models found for this variable are not recommended for predictive calculations; this lack of fit may be due to measurement errors or because the fermentation dynamics of yeasts are more complex, and the models may be influenced by operational variables not considered in the experimental design of this study.

Amounts of EtOH between 2.14 and 4.35 g/L have been reported in an aerobic environment at 21 °C (Laureys *et al.,* 2017). On the other hand, Teixeira in 2009 reported amounts up to 1.2 g/L in aerobic fermentations with WKG at 25°C. It can be stated that the fermentation conditions of this study favored

the formation of ethanol since an anaerobic atmosphere was created and the temperatures evaluated are conducive to the activity of yeasts, which are the main producers of this compound.

EtOH varied from 7 to 25.5 g/L for WKGCOL3 and from 7 to 21 g/L for WKGCH2, with the lowest ethanol values for both grains occurring in test 8 (20 °C and 80 rpm). However, in the metabarcoding analysis, the yeasts showed the highest abundances at the lower temperatures (20 and 23 °C), which may indicate that there are significant differences in the environmental requirements of the six different species identified. Given that increasing the temperature (experiment 3, 37°C and 137 rpm) seems to favor the production of ethanol in both grains, it is important to operate at a condition of 20°C to prevent the resulting beverages from having a significant alcohol content and to promote a prototype beverage that is not classified as an alcoholic product. Figures 4.10d and 4.11d show the quadratic behavior fitted to the data obtained from the ethanol measurement.

4.5.5. Acetic acid (AA) formation in water kefir fermentation

For WKGCH2, the quadratic regression equation had a significant p-value of 0.5905. On the other hand, WKGCOL3 also fitted a quadratic regression with a p-value of 0.3703. The R^2 for both grains are less than 0.4, indicating a low correlation between the response variable and the factors. Based on the statistics evaluated, the use of these models for predictive purposes is not recommended. Since the models adjusted for EtOH did not show good fits, it is only obvious that the models for AA will also have fit problems, knowing that the formation of this metabolite is closely linked to the formation of ethanol. According to the data, for this variable in WKGCOL3, the maximum value was 0.95 g/L in experiment 3 (137 rpm and 37°C), and the minimum value was 0.40 g/L in treatment 8 (80 rpm and 20°C). For WKGCH2, the maximum value of AA was 0.7 g/L in experiment 3 (137 rpm and 37°C) and the minimum value was 0.15 g/L in treatment 8 (80 rpm and 20°C). These data show that at the lowest temperature used in this design (20°C), the amount of this acid decreases; this also occurs in the case of ethanol and reinforces the close relationship between the formation of both metabolites. It can also be concluded that the amount of acetic acid for both grains is minimal (>0.1%) in practically all treatments, so its presence in the final product does not have a significant effect on its flavor and aroma.

Laureys *et al.,* (2017) reported acetic acid (AA) levels of up to 0.4 g/L in an aerobic process, and Texeira *et al.,* (2010) reported AA levels of 1.2 g/L in the same type of process. In comparison with this study, the maximum AA levels are like those reported by Laureys, although it should be noted that the fermentation times in this study are longer, and therefore the anaerobiosis and configuration of the

fermentations in this study have an inhibitory effect on the growth of acetic bacteria. In the present study, for grain WKGCOL3, ranges of this organic acid were observed between 0.4 and 0.95 g/L, and they do not represent significant changes between treatments, which is reflected by the lack of fit to any equation, as shown in Figure 4.11c. For the grain WKGCH2, ranges of AA formation between 0.15 and 0.70 g/L were observed, where a small variation in the amount of this compound is also reflected in the data set and therefore the fitting of a regression equation is also affected. (Figure 4.11c). For both grains, it was observed that in experiment 3 (37°C and 137 rpm), there was a slight increase in the amount of AA, while in experiment 8 (20°C and 80 rpm), the formation of this metabolite was reduced. It is likely that the LAB contributes to the increase of AA since the higher values of LA are also present in experiment 3. It can also be observed that the biomass growth for both grains in condition 3 is low compared to the other experiments. This is inversely proportional to the production of AA and LA, so it can be concluded that these organic acids may negatively affect the growth of the grains, as stated by Laureys *et al.,* 2016.

4.5.6. Sugar consumption in water kefir fermentations

For GLUC, second order regressions were found in both grains; in WKGCH2, the equation had a p-value of 0.3681 and the R^2 value was 0.4397. The equation cannot be used to predict the effects of the factors according to the last parameter. On the other hand, for WKGCOL3, GLUC fitted with a significant value of p=0.5767, and the R^2 was 0.3347, indicating a low relationship between the response variable and the factors. It is also concluded that it is not recommended to be used for prediction purposes. For WKGCH2, FRUC was fitted to a quadratic equation with a p-value of 0.126, and in WKGCOL3 it was also fitted to a quadratic equation with a p-value of 0.2795. In WKGCH2, the R^2 was found to be 0.6037 and 0.4903 for WKGCOL3, indicating a better fit for WKGCH2. The CV for WKGCH2 indicates that the equation shows no significant deviations and a good relationship between the factors and the response; therefore, it is only recommended that the model found for WKGCH2 be used for predictive purposes in other similar processes. In the results for SUCR, we found that for WHGCH2, a linear equation was fitted with a significant p-value of 0.0461 and an R^2 of 0.53, indicating a lack of model fit. In the case of WKGCOL3, a quadratic equation was fitted with a p-value of 0.3883 and the coefficient of determination R^2 was 0.4289, also indicating that the response variables are not fully represented by the equation. Therefore, the use of these models is not recommended.

The amounts of GLUC, FRUC and SUCR at the end of fermentation. In each treatment, the maximum and minimum sugar contents are very similar for both grains, which also indicates clear trends and common behavioral patterns between both grains. The chromatographic (HPLC) analysis showed that

the total sugars of NCS used for the fermentations started from a substrate concentration of 80 g/L, of which the SUCR content was 61.93 g/L, GLUC 12.81 g/L and FRUC 2.85 g/L. For WKGCOL3, GLUC varied between 2.15 and 11.65 g/L for experiments 3 (37.07°C and 136.57 rpm) and 8 (30°C and 80 rpm), with a consumption between 17% and 91%; FRUC varied between 3.90 and 11.45 g/L for experiments 7 (22.93°C and 23. 43 rpm) and 4 (40 °C and 80 rpm), respectively, which showed that FRUC was not consumed, but rather increased as the hydrolysis of sucrose progressed (Teixeira *et al.*, 2010), reaching values up to 401% of the initial content; SUCR was between 6.35 and 61.90 g/L for experiments 3 (37.07°C and 136.57 rpm) and 8 (30°C and 80 rpm), just like GLUC, with a consumption percentage of up to 99%, showing the relationship between SUCR and GLUC and possibly indicating that the hydrolysis of SUCR is directly proportional to the consumption of GLUC.

For WKGCH2, GLUC varied between 4.15 and 12.80 g/L for experiments 7 (22.93 °C and 23.43 rpm) and 8 (30 °C and 80 rpm), respectively, while FRUC varied between 8.75 and 16.50 g/L for experiments 2 (30 °C and 160 rpm) and 4 (40 °C and 80 rpm), respectively, and SUCR varied between 11.00 and 48.55 g/L for experiments 4 and 8, respectively. FRUC was also not consumed, but rather increased as the hydrolysis of sucrose progressed, reaching values up to 579%. Sucrose is the most abundant sugar in the NCS, accounting for more than 80%. It is possible that when the kefir micro-organisms consume all the free and available glucose and fructose in the NCS, they begin to hydrolyze the sucrose, producing very high levels of fructose and glucose (even higher than at the start of fermentation). The consumption of GLUC was 78.4%, confirming that for both grains this compound is preferentially used by microorganisms as an energy source. The condition that best meets a sugar content of less than 50 g/L (according to Chilean labeling regulations and free of seals) for both fermentations is condition 3 (37°C, 136.57 rpm), where the highest total sugar consumption was observed. According to this restriction, the conditions that meet this regulatory aspect are 3, 4 and 7, with minimum final total sugars of 14.75 g/L for WKGCOL3 and 26.95 g/L for WKGCH2. In general, the total sugar content indicates that the initial concentration of NCS can be lower to ensure compliance with the standard.

4.5.7. Regressing Polynomials obtained by RSM

The summary of the coefficients of the polynomials obtained for all the response variables in relation to the factors is shown in Table 4.8 below. The polynomials and results obtained under each condition could be used for prediction and search for the desired results according to the goodness of fit found for each model. The limits for optimization purposes are defined according to the numerical range of the results, considering that these limits are reasonable within the logic of the experiment itself, a range

where the response variable can move from one limit to another, and its significance is the weight assigned to each response variable based on certain acceptable physicochemical characteristics.

Table C (Appendix 8.5) describes the levels of importance of the responses and their desired ranges. The goal is to find polynomial relationships to predict the value of the response variables. Values are assigned from 1 (+) to 5 (+++++) for the importance scale.

Table 4.8. *The empirical models obtained for each response variable in each grain are presented in terms of factors (X1 rpm, X2 °C) and regression coefficients bi. The order of each equation is determined by the degree of the factors; for both grains, first and second-degree polynomial equations were presented.*

	WKGCOL3	***Full empirical models***						
Factor	***Regression Coefficient***	***Final grain (FG) g/L***	***Lactic acid (LA) g/L***	***Acetic acid (AA) g/L***	***Ethanol (EtOH) g/L***	***Glucose (GLUC) g/L***	***Fructose (FRUC) g/L***	***Sucrose (SUCR) g/L***
	Linear							
	b_0	*114,732*	*18,19501*	*0,937447*	*42,64641*	*-9,47043*	*22,78291*	*-99,13461*
X_1 ***-rpm***	b_1	*-0,98989*	*-0,089854*	*-0,011231*	*-0,185928*	*0,184098*	*0,074762*	*0,861019*
X_2 ***-°C***	b_2	*4,17097*	*-1,0889*	*0,00662*	*-1,90846*	*0,771536*	*-1,43458*	*8,27604*
	Quadratic							
X_1*X_2	***b3***	*0,014502*	*0,002032*	*0,000156*	*0,006563*	*-0,005282*	*-0,002438*	*-0,024441*
X_1^2	***b4***	*2,94E-03*	*2,05E-04*	*4,20E-05*	*6,30E-05*	*-2,03E-04*	*7,20E-05*	*-1,20E-03*
X_2^2	***b5***	*-0,110325*	*0,020652*	*-0,000313*	*0,029017*	*-0,007252*	*0,028092*	*-0,121003*
	WKGCH2	***Full empirical models***						
Factor	***Regression Coefficient***	***Final grain (FG) g/L***	***Lactic acid (LA) g/L***	***Acetic acid (AA) g/L***	***Ethanol (EtOH) g/L***	***Glucose (GLUC) g/L***	***Fructose (FRUC) g/L***	***Sucrose (SUCR) g/L***
	Linear							
	b_0	*43,20684*	*13,32497*	*-0,691458*	*15,05837*	*1,19314*	*45,15297*	*14,45712*
X_1 ***-rpm***	b_1	*-0,688101*	*-0,118478*	*-0,004275*	*-0,052037*	*0,150712*	*-0,01113*	*0,620134*
X_2 ***-°C***	b_2	*6,9007*	*-0,647811*	*0,084545*	*-0,246316*	*0,241519*	*-2,36126*	*0,461369*
	Quadratic							
X_1*X_2	***b3***	*0,01256*	*0,001906*	*0,000188*	*0,004376*	*-0,003719*	*-0,000063*	*-0,020034*
X_1^2	***b4***	*1,61E-03*	*3,70E-04*	*-5,27E-06*	*-4,54E-04*	*-2,47E-04*	*2,50E-05*	
X_2^2	***b5***	*-0,143426*	*0,013921*	*-0,001588*	*0,003459*	*-0,001796*	*0,040383*	

$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2 + b_4X_1^2 + b_5X_2^2$; X_1 = Agitation; X_2 = Temperature

4.5.8. Operating Point for Fermentation Operating Conditions.

Table 4.9 and Figure 4.7 show the predicted point that was found to be the best solution according to the desirability function, calculated as Global Desirability (GD), which was 0.578 for WKGCOL3, with the optimal operating point being agitated (65.7 rpm) and temperature (37.07°C). Table 4.10 shows the expected predicted values for each response at the operating point.

Table 4.8 shows the fits of the empirical model for WKGCH2. Table 4.11 and Figure 4.8 show the prediction point that was found to be the best solution according to the GD function, which was 0.602. The GD confirms that the operating point found is very close to the predicted desirability value (~1), with the operating point being the agitation (23.43 rpm) and the temperature (37.07°C).

Table 4.9. *The WKGCOL3 prediction points of the design (level), the standard deviation, the coding of the factors and the values of the levels used in the design can be observed.*

Factor	Name	Level	Low Level	High Level	Std. Dev.
X_1	**Agitation (rpm)**	65,7	23,43	136,57	0
X_2	**Temperature (°C)**	37,07	22,93	37,07	0

Table 4.10. *Possible solutions for each response variable according to the models constructed for WKGCOL3 experiment, the standard deviation, and its confidence interval.*

Solution Response	Predicted Mean	Std Dev	95% CI low for Mean	95% CI high for Mean
FG	100,726	8,41113	90,5434	110,909
LA	6,14016	0,907973	5,04095	7,23936
AA	0,577246	0,15741	0,386683	0,767809
EtOH	15,8148	4,413	10,4723	21,1572
GLUC	7,5189	2,53539	4,44951	10,5883
FRUC	7,49003	1,7697	5,34761	9,63245
SUCR	33,2641	14,0839	16,2139	50,3143

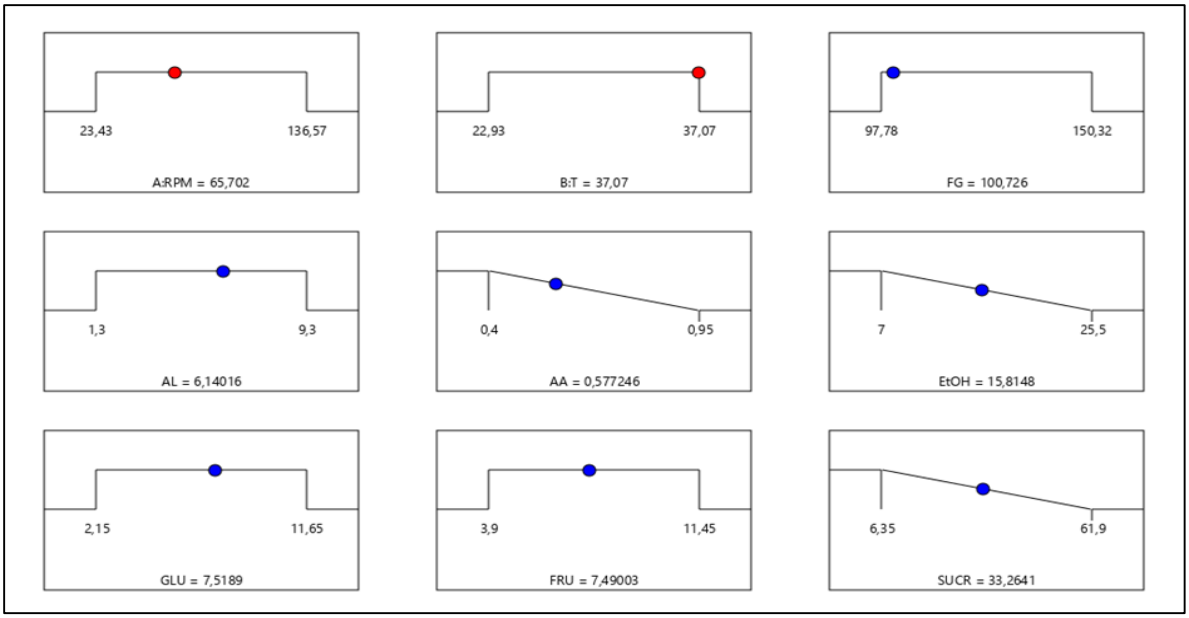

Figure 4.7. *Numerical solution for WKCOL3 observed in ramp plots according to the constraints used. The values at the ends of the ramps are the limits used to decrease or increase the sample range; the value at the bottom of each ramp corresponds to the predicted value for each response variable. The red dots represent the factors, and the blue dots represent the responses.*

Table 4.11. *The WKGCH2 prediction points of the design (level), the standard deviation, the coding of the factors and the values of the levels used in the design can be observed.*

Factor	Name	Level	Low Level	High Level	Std. Dev.
X_1	**Agitation (rpm)**	23,43	23,43	136,57	0
X_2	**Temperature (°C)**	37,07	22,93	37,07	0

Table 4.12. *Possible solutions for each response variable according to the models constructed for WKGCH2 experiment, the standard deviation, and its confidence interval.*

Solution Response	Predicted Mean	Std Dev	95% CI low for Mean	95% CI high for Mean
FG	97,5904	11,9949	75,723	119,458
LA	7,52342	1,2706	5,20704	9,8398
AA	0,320267	0,14646	0,0532609	0,587272
EtOH	13,0133	3,90225	5,8993	20,1273
GLUC	7,84308	1,96297	4,26447	11,4217
FRUC	12,8138	1,65726	9,79248	15,8351
SUCR	28,6895	8,27362	14,7541	42,6249

Table 4.12 shows the expected predicted mean values for each response, the standard deviation, and the confidence interval within which the predictions of the equations can vary, according to the polynomials chosen and the constraints used. Figure 4.8 shows the solution in ramp plots.

The expected values for FG, AA, EtOH, GLUC, FRUC, and SUCR are within the range expected in the final beverage given the restrictions imposed. Figures 4.10 and 4.11 show the response surfaces found for each variable, where the results can be observed according to the influence of temperature and agitation. The curvatures indicate the non-linearity of the fitted polynomials. The red dots are the values for the experiments that are above the surface (i.e., outside the range calculated for the regression equations), and the pink dots are those that are also outside the range but below the response surface. The color of the graphs indicates the highest and lowest values, with blue shading indicating the lowest values and red shading indicating the points with the highest values. FRUC, among all the sugars analyzed, has an inverted surface with respect to SUCR and GLUC, confirming that its behavior is subject to different conditions. The same was observed for FG, the highest growth was observed at the lowest temperatures, but also at the lowest LA and AA contents.

The operating conditions found for both grains, 66 rpm - 37 °C for WKGCOL3 and 23 rpm - 37 °C for WKGCH2, are operating points that coincide in temperature. Although the agitation to be applied is not the same, it can be said that since these are experiments evaluated in an orbital shaker and considering the clustered nature of the grains, these agitation values are so low that they do not represent noticeable changes in the movement of the fermentation system during the process, a factor that begins to be

distinguishable at agitation levels above 80 rpm. This last point is based on what was observed in each of the experiments carried out.

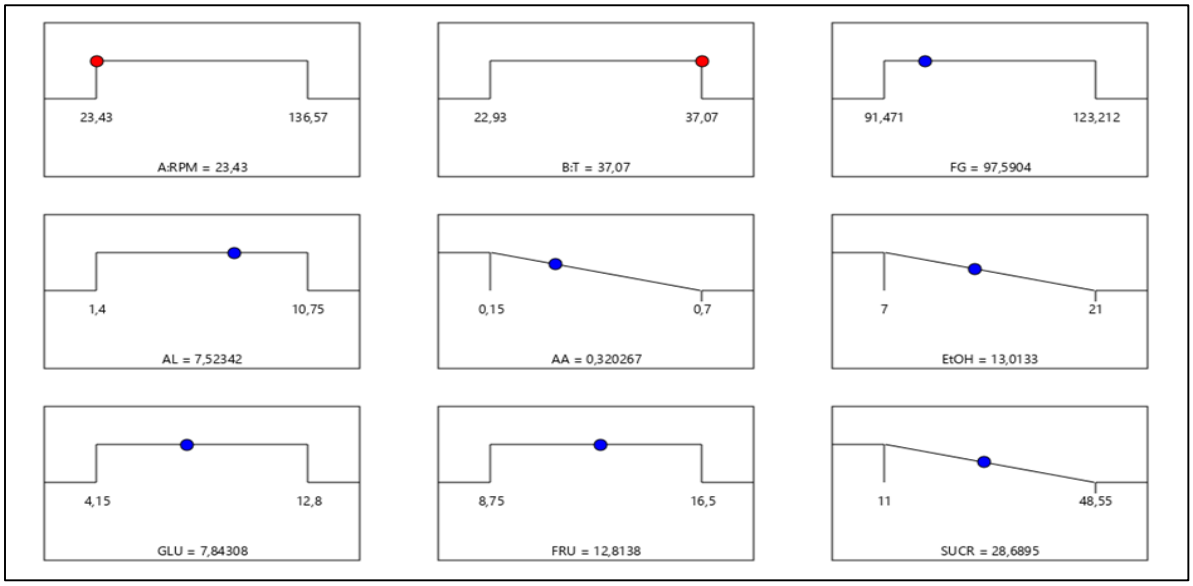

Figure 4.8. *Numerical solution for WKCCH2 observed in ramp plots according to the constraints used. The values at the ends of the ramps are the limits used to decrease or increase the sample range; the value at the bottom of each ramp corresponds to the predicted value for each response variable. The red dots represent the factors, and the blue dots represent the responses.*

A general optimization was carried out for both grains using the grouped data and the same restriction criteria described in Table 4.13, and it was found that the operating point coincides at a temperature of 37°C and at a low agitation of 38 rpm, with an acceptable GD of 0.552 (Figure 4.9). Therefore, the operating point for obtaining a standard beverage with any of the studied kefir grains and with the desired characteristics according to the contents of ethanol, acids, sugars, and biomass increase was established at 37°C and 38 rpm. Given that this study was conducted with sufficient robustness and based on two native grains from Latin America of different origins, it can be determined that these conditions and the models with significant adjustments can be used to evaluate and develop fermentations with WKG from other origins, considering that the process must be carried out under anaerobiosis and at initial concentrations of inoculum and substrate of 8 g/L. Table 4.14 shows the expected predicted mean values for each response in the global optimization, the standard deviation, and the confidence interval within which the predictions of the equations can vary, according to the polynomials chosen and the constraints used.

Table 4.13. *The general prediction point (level) for optimization can be observed with both grains (WKGCOL3 and WKGCH2), the standard deviation, the coding of the factors and the minimum and maximum values considered.*

Factor	Name	Level	Low Level	High Level	Std. Dev.
X_1	**Agitation (rpm)**	38.14	23.43	136.57	0.000
X_2	**Temperature (°C)**	37.07	22.93	37.07	0.000

Table 4.14. *Possible solutions for each response variable according to the models constructed for with both grains (WKGCOL3 and WKGCH2), the standard deviation, and its confidence interval.*

Solution Response	Predicted Mean	Std Dev	95% CI low for Mean	95% CI high for Mean
		WKGCOL3		
FG	104,7724283	5,493213518	92,10505533	117,4398013
LA	5,953228865	0,592986649	4,585799211	7,320658518
AA	0,606965554	0,102802591	0,369902355	0,844028753
EtOH	14,05400908	2,882076303	7,407929266	20,70008889
GLUC	8,422847846	1,65583663	4,604481765	12,24121393
FRUC	7,715266209	1,155768597	5,05005907	10,38047335
SUCR	40,42508128	8,8304908	20,74952171	60,10064085
		WKGCH2		
FG	95,7710899	7,832740091	77,70875902	113,8334208
LA	7,15517451	0,829817253	5,241612511	9,068736509
AA	0,354850508	0,095651616	0,134277489	0,575423527
EtOH	14,2226729	2,548515305	8,345786118	20,09955967
GLUC	7,808461321	1,281994776	4,852176093	10,76474655
FRUC	12,63893095	1,082340448	10,14304943	15,13481248
SUCR	26,88758328	5,244379905	15,2023767	38,57278986

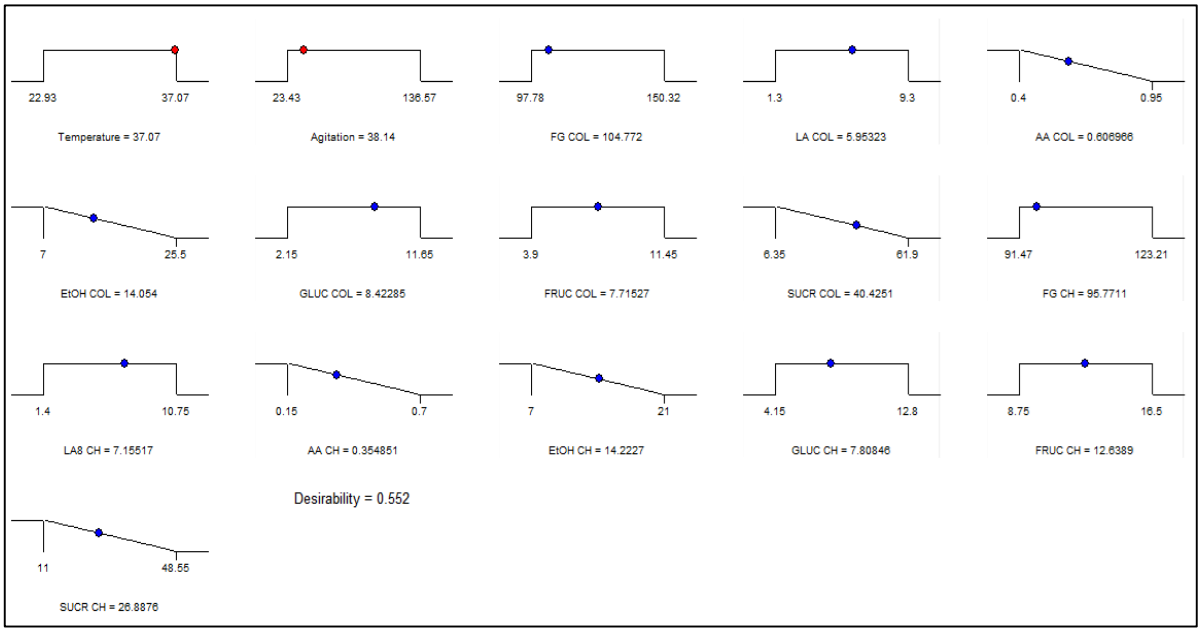

Figure 4.9. *Numerical solutions for with both grains (WKGCOL3 and WKGCH2) observed in ramp plots according to the constraints used. The values at the ends of the ramps are the limits used to decrease or increase the sample range; the value at the bottom of each ramp corresponds to the predicted value for each response variable. The red dots represent the factors, and the blue dots represent the responses.*

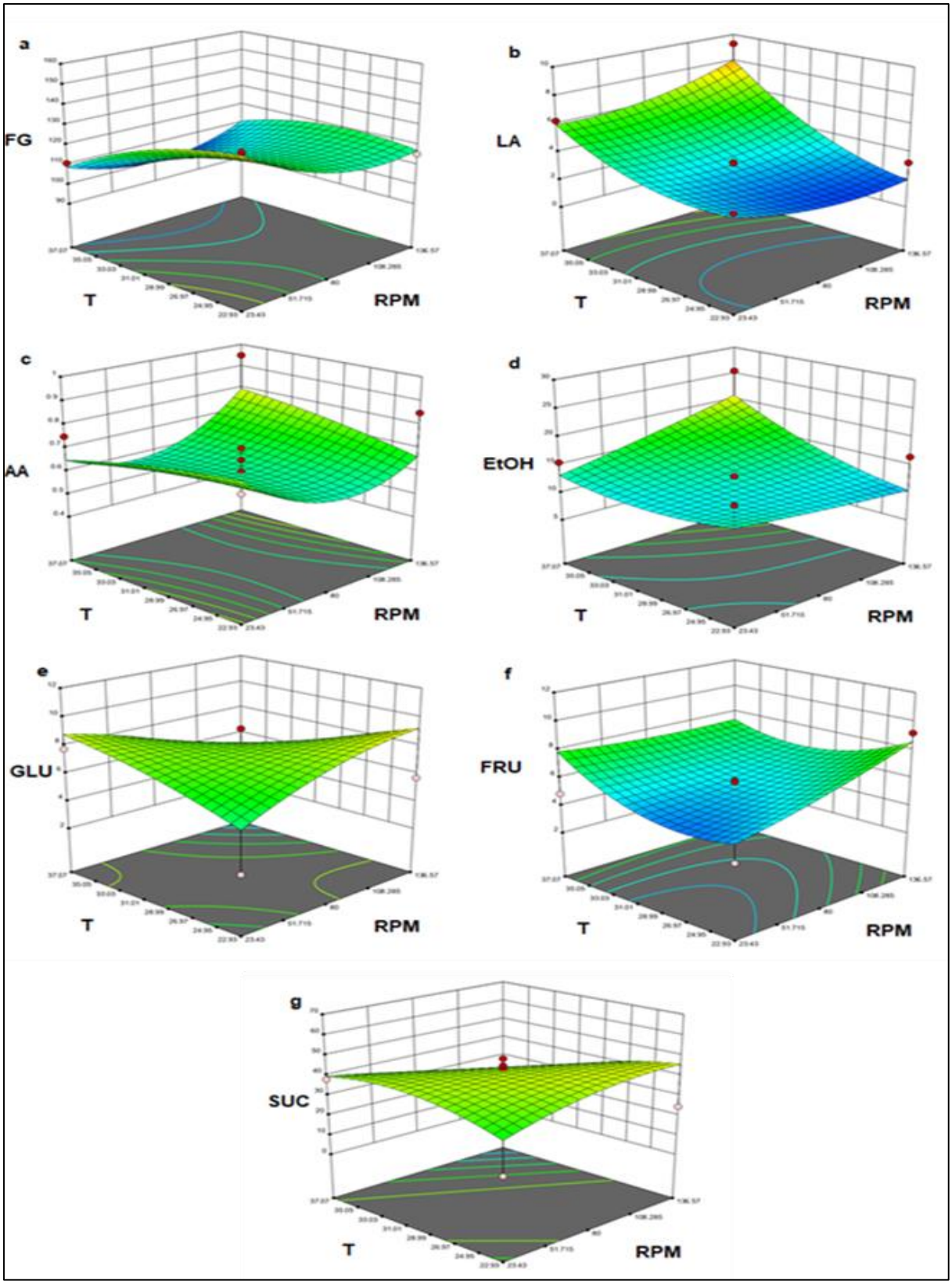

***Figure 4.10**. Colombian water kefir response surface plots (3D). The effects of Temperature (T) and Agitation (RPM) on (a) Final grain - FG; (b) Lactic acid - LA; (c) Acetic acid - AA; (d) Ethanol - EtOH; (e) Glucose - GLU; (f) Fructose – FRU; (g) Sucrose – SUC. All results for variables are reported in g/L.*

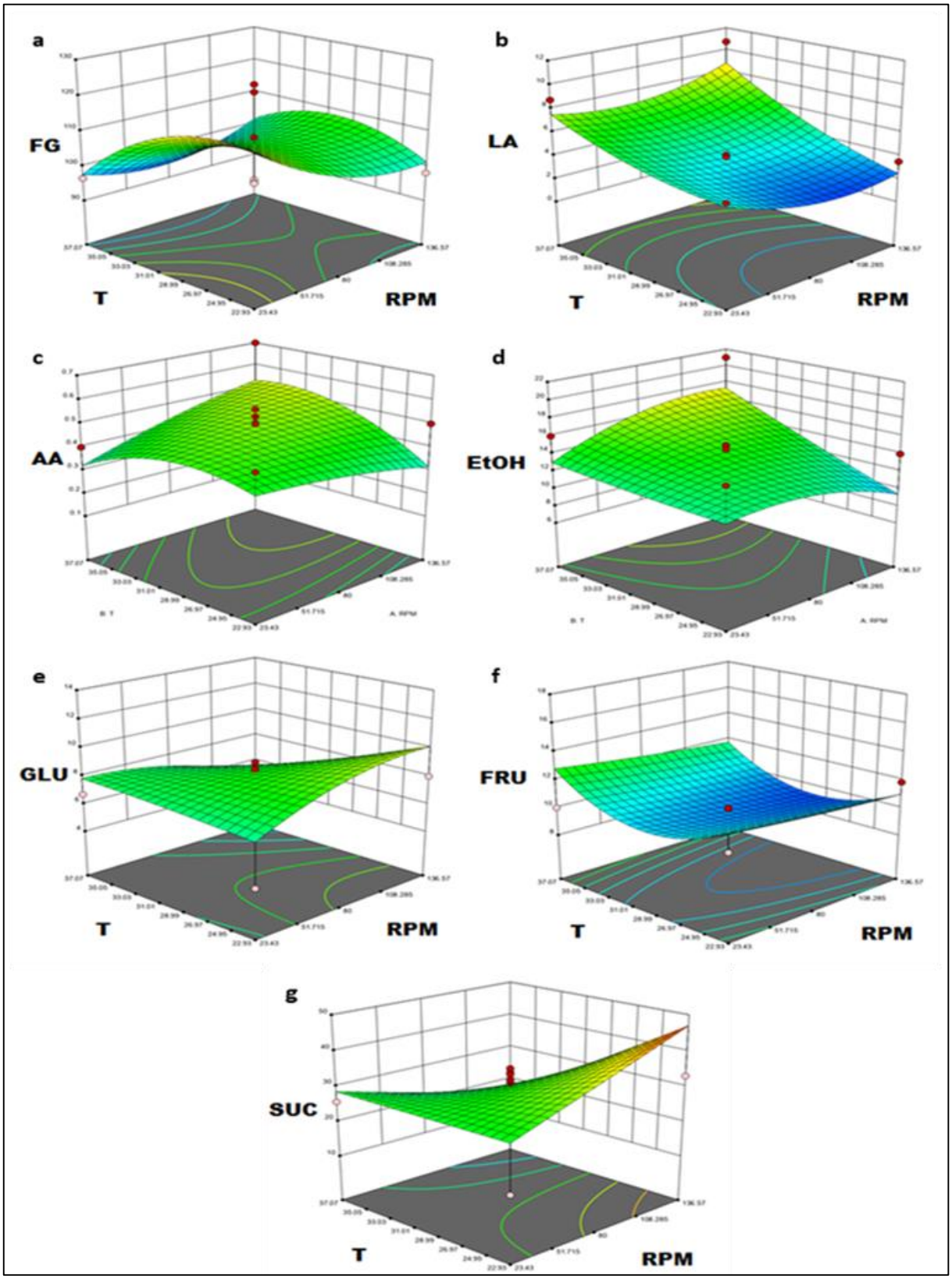

Figure 4.11. *Chilean water kefir response surface plots (3D), experiment at 500 mL. The effects of Temperature (T) and Agitation (RPM) on (a) Final grain - FG; (b) Lactic acid - LA; (c) Acetic acid AA; (d) Ethanol - EtOH; (e) Glucose - GLU; (f) Fructose – FRU; (g) Sucrose – SUC. All results for variables are reported in g/L.*

4.6. Determination of optimal operating conditions for water kefir grain fermentations using Response Surface Methodology (Experiment at 250 ml).

The results obtained at 250 ml are shown in Tables 4.15 and 4.16. For WKGCOL3, the minimum amount of biomass (FG) obtained in all experiments was 99.97 g/L and the maximum was 123.48 g/L. Regarding the metabolites produced, LA varied between 1.24 and 11.34 g/l, AA between 0.26 and 0.93 g/L, and EtOH had a minimum of 9.21 and a maximum of 20.42 g/L; for sugars, GLUC varied between 3.83 and 10.70 g/L, FRUC had a minimum of 4.99 and a maximum of 19.67 g/L, and SUCR varied between 8.16 and 35.72 g/L. For WKGCH2, FG varied from 92.81 to 121.17 g/L, LA from a minimum of 2.17 to a maximum of 11 g/L, AA from 0.22 to 0.62 g/L, EtOH from 7.62 to 19.57 g/L, GLUC from 4.97 to 11.97 g/L, FRUC from 6.43 to 23.40 g/L, and SUCR from a minimum of 7.97 to a maximum of 38.95 g/L. The standard deviation (SD) was taken as an indicator of the possible reproducibility of the experiment under certain operating conditions and the use of different polynomials for predictive purposes, based on an analysis of the deviation of the data from the sample means in the different groups of experiments conducted for each grain (WKGCOL3 and WKGCH2) and for each scale. (500 and 250 mL). Table D (Appendix 8.5) shows the standard deviations for each grain and at a general level, calculated from the data under each operating condition.

For FG, it was found that at a temperature of 30°C, the highest and lowest grain increases were recorded at stirring speeds of 160 and 23 rpm, respectively, which may indicate that stirring can affect the growth of WKG. It can also be observed that at temperatures of 23 and 30 °C, the highest grain growth was recorded, with values up to 123 g/L for WKGCOL3 and up to 122 g/L for WKGCH2. In contrast, at the higher temperatures of 37 and 40 °C, the lowest growths were observed, with values up to 100 g/L for WKGCOL3 and up to 93 g/L for WKGCH2. The above is confirmed by the fact that the microbial groups present in the grains that are more involved in grain growth (lactic acid bacteria - LAB) have optimal growth temperatures close to 30°C. Furthermore, in the optimization carried out on the metabarcoding data, it was found that the species of lactic acid bacteria that produce dextran, which are the most abundant, were present at temperatures between 23 and 30°C. One of the operating conditions that showed the greatest reproducibility in the response variable FG was that conducted at 80 rpm and 30°C, where the standard deviations deviated by less than 10% from the sample mean. In addition to the last condition, experiments 3 (137 rpm and 37°C) and 4 (80 rpm and 40°C) had the lowest overall standard deviations, with values deviating less than 5% from the calculated mean. In general, for FG, all operating conditions had standard deviations between 3.9% and 11.7% relative to the calculated sample means, which may indicate that this experimental design is reproducible at the scales evaluated

(500 and 250 mL) and that the calculated polynomials could also be used as approximate prediction standards.

On the other hand, in condition 3 (137 rpm and 37°C), LA was mainly produced in both grains with values up to 11 g/L, while the lowest amounts were observed in experiment 8 (80 rpm and 20°C) with values between 1.2 and 1.6 g/L (WKGCOL3 and WKGCH2 respectivily). These results are related to the fact that the bacteria that are the main producers of LA are the LAB, which have optimal temperatures close to 37°C. This shows that the experiments are reproducible at the scales evaluated and that the expected amounts of metabolites vary little even when grains from different sources are used. AA showed minimal levels in condition 8 (80 rpm, 20°C), with values up to 0.26 g/L for WKGCOL3 and up to 0.22 g/L for WKGCH2; this condition was also unfavorable for the formation of LA, as previously described, which may indicate a direct relationship between the metabolism of lactic and acetic acid bacteria or microorganisms capable of producing both acids. In turn, the maximum levels of AA were observed in condition 9 (80 rpm, 30°C) for WKGCOL3 and in condition 2 (160 rpm, 30°C) for WKGCH2, with amounts up to 1.01 and 0.62 g/L, respectively. This is also confirmed by the fact that LAB and AAB (acetic acid bacteria) have optimal temperatures above 30°C. In all the experiments, it was observed that the AA content was less than 0.01% of the total fermentation volume, which means that the anaerobic atmosphere effectively prevents the formation of this acid, identified as a factor of low quality.

The EtOH had maximum values in condition 2 and minimum values in condition 8, with up to 21 g/L for WKGCOL3 and up to 20 g/L for WKGCH2 (as maximum amounts) and up to 8 g/L for both grains as minimum amounts, with these conditions showing low significant standard deviations and less than 13% deviation from the overall mean of the sample. The condition at 80 rpm and 30°C also shows reproducibility as the standard deviations are less than 7% of the overall mean and the EtOH contents found in all replicates are very similar across all data sets. In conclusion, the response variables FG, LA, AA and EtOH show reproducibility in the experimental design and present insignificant standard deviations, indicating that the experiment can be replicated at other times and for WKG from other sources.

Regarding sugar consumption, it was observed that for GLUC, condition 8 (80 rpm, 20°C) had the lowest uptake among the four experimental groups, with up to 10.7 g/L for WKGCOL3 and 12 g/L for WKGCH2. At the same time, the highest uptake of GLUC occurred in condition 2, with up to 3.83 g/L for WKGCOL3 and up to 4.97 g/L for WKGCH2. As can be seen, conditions 2 (160 rpm, 30°C) and 8 (80 rpm, 20°C) show the highest and lowest values to produce the different metabolites, biomass

formation and sugar consumption. The consumption of FRUC was lowest in both grains in condition 4 (80 rpm, 40°C) with values of 19.7 g/L for WKGCOL3 and 23.4 g/L for WKGCH2; the highest consumption was recorded in condition 2 (160 rpm, 20°C) with values up to 4.99 and 6.43 g/L for WKGCOL3 and WKCH2, respectively. This monosaccharide behaved differently in the other conditions, confirming that microorganisms prefer to consume GLUC, which slows down the metabolism of FRUC.

Finally, the SUCR the maximum consumption was observed in condition 4 (80 rpm, 40°C) for WKGCH2 and condition 3 (136 rpm, 37°C) para WKGCOL3, with amounts up to 8.2 g/L for WKGCOL3 and 8 g/L for WKGCH2, while the minimum consumption was recorded at 36 g/L for the Colombian grain and 39 g/L for the Chilean grain.

It is undeniable that temperature has a strong influence on the final results of fermentation, although in some experiments, such as FG, agitation seems to favor the increase of biomass, as reported by Scheoevers *et al.*, (2003), who observed that agitation, together with the addition of other nutrients, improved growth, consortium activity and acid production; this was also the case in the present experiment, where at 137 rpm the highest biomass yields, the greatest metabolite production and the highest sugar consumption were observed.

In summary, it can be observed that in this experiment carried out at 250 mL, the values obtained for the response variables are close to those obtained at 500 mL, except for FRUC and SUCR, where the values obtained for FRUC at 250 mL are almost double those obtained at 500 mL, and the opposite is true for SUCR. This may also be because agitation and fermentation scale play an important role in the homogeneous access of the WKGs to the substrate, as explained above. However, it is observed that the separate experiments for these two response variables could be reproducible according to the standard deviations obtained. Conditions 3 and 8 are also defined as those that maximize or minimize the production of biomass and metabolites. It is also noted that the evaluated workload indicates that no significant changes are observed.

4.6.1. Evaluation of the effect of operating conditions using Response Surface Methodology

The combined effect of fermentation temperature (°C) and stirring (rpm) on fermentation, FG (g/L), LA (g/L), AA (g/L), GLUC (g/L), FRUC (g/L) and SUCR were evaluated. It was determined by RSM - CCD (Table 3.3), whose experimental results are shown in Tables 4.15 and 4.16. The results of the

analysis of variance are shown in Table E (Appendix 8.5) ANOVA; the final predictive equations were formed from the data and test statistics described in this table. Among the 8 proposed mathematical equations, some of the responses could be appropriately estimated through the analysis of variance for each of the response variables to consider the fit. The data obtained and their interpretation and analysis are presented next.

Table 4.15. *Measured data for the response parameters in the fermentation of WKGCOL3 (250 mL), final grain (FG), lactic acid (LA), acetic acid (AA), ethanol (EtOH), glucose (GLUC), fructose (FRUC), and sucrose (SUCR), respectively. Data reported are in g/L.*

Run	Agitation RPM (X_1)	Temperature °C (X_2)	Exp. Code	FG (Y_1)	LA (Y_2)	AA (Y_3)	EtOH (Y_4)	GLUC (Y_5)	FRUC (Y_6)	SUCR (Y_7)
				(g/L)						
1	136.57	22.93	COL3E1	119.62	2.42	0.67	16.36	6.11	7.57	23.18
2	160.00	30.00	COL3E2	123.48	4.89	0.81	20.42	3.83	4.99	14.77
3	136.57	37.07	COL3E3	101.68	11.34	0.69	19.57	4.07	10.82	8.16
4	80.00	40.00	COL3E4	100.44	6.50	0.44	14.81	8.26	19.67	10.71
5	23.43	37.07	COL3E5	99.97	8.58	0.49	18.78	4.73	10.56	10.96
6	0.00	30.00	COL3E6	116.00	3.26	0.63	18.99	4.26	8.76	14.08
7	23.43	22.93	COL3E7	120.20	2.20	0.54	15.29	6.59	11.38	21.65
8	80.00	20.00	COL3E8	112.240	1.24	0.26	9.21	10.70	11.43	35.72
9	80.00	30.00	COL3E13	111.144	4.78	1.01	15.25	6.35	10.14	18.14
10	80.00	30.00	COL3E13.1	107.54	4.67	0.61	14.86	6.58	10.96	18.38
11	80.00	30.00	COL3E13.2	109.342	4.72	0.81	15.05	6.47	10.55	18.26
12	80.00	30.00	COL3E13.3	104.12	4.69	0.93	15.00	6.34	10.15	18.42
13	80.00	30.00	COL3E13.4	108.22	4.87	0.70	15.62	6.55	10.89	18.27
14	80.00	30.00	COL3E 13.5	111.87	4.75	0.63	14.93	6.39	10.68	18.43

Table 4.16. *Measured data for the response parameters in the fermentation of WKGCH2 (250 mL), final grain (FG), lactic acid (LA), acetic acid (AA), ethanol (EtOH), glucose (GLUC), fructose (FRUC), and sucrose (SUCR), respectively. Data reported are in g/L.*

Run	Agitation RPM (X_1)	Temperature °C (X_2)	Exp. Code	FG (Y_1)	LA (Y_2)	AA (Y_3)	EtOH (Y_4)	GLUC (Y_5)	FRUC (Y_6)	SUCR (Y_7)
				(g/L)						
1	136.57	22.93	CH2EXP1	121.17	2.17	0.33	12.75	7.13	10.11	26.87
2	160.00	30.00	CH2EXP2	102.25	6.15	0.62	19.57	4.97	6.43	18.00
3	136.57	37.07	CH2EXP3	94.17	11.00	0.45	19.17	4.99	12.75	9.06
4	80.00	40.00	CH2EXP4	93.84	7.15	0.30	12.80	9.72	23.40	7.97
5	23.43	37.07	CH2EXP5	92.81	10.21	0.49	17.48	5.31	12.47	10.92
6	0.00	30.00	CH2EXP6	106.31	3.54	0.51	17.78	5.51	12.44	14.18
7	23.43	22.93	CH2EXP7	120.26	1.98	0.33	11.25	7.51	13.86	23.73
8	80.00	20.00	CH2EXP8	106.96	1.19	0.22	7.62	11.97	12.46	38.95
9	80.00	30.00	CH2EXP13	100.064	4.91	0.41	14.05	7.98	12.97	19.93
10	80.00	30.00	CH2EXP13.2	99.54	4.78	0.34	14.15	7.78	13.29	18.66
11	80.00	30.00	CH2EXP13.3	99.80	4.84	0.37	14.10	7.88	13.13	19.29
12	80.00	30.00	CH2EXP13.4	98.62	4.86	0.39	14.20	7.95	13.34	19.85
13	80.00	30.00	CH2EXP13.5	99.95	4.75	0.38	13.80	7.76	13.10	18.98
14	80.00	30.00	CH2EXP13.6	98.88	4.83	0.40	14.00	7.54	13.25	19.34

4.6.2. Final Grain (FG) Operating Conditions

According to the results found in ANOVA Table E (Appendix 8.5), the fitted polynomial for WKGCOL3 was quadratic, with a p-value of 0.0055, indicating that all factors are not significant for the polynomial regression. The correlation coefficient R^2 was 0.83, this criterion also indicates that the FG variable is represented by the polynomial. For WKGH2, the polynomial found in the ANOVA was linear and showed a good fit. The R^2 was 0.70, indicating that the data fit the selected equation and is consistent with goodness of fit. For WKGCOL3, the fit is also linear with an R^2 value of 0.83, indicating that the polynomial is also appropriate.

According to the results, the biomass increases were slightly higher for the WKGCOL3 grain. It is worth noting that treatment 5 was common to both grains in terms of reduced growth, which means that perhaps at a temperature of 37°C, the growth of dextran is reduced to a greater extent. This is consistent with what was previously evaluated for the 500 mL experiments and explains the relationship between the abundance of *Lactobacillus* species and the formation of this polymer at temperatures between 23 and 30°C.

For WKGCOL3, the treatment with the best grain increase was at 160 rpm and 30°C, with 43.48 g and a yield percentage of 171%. On the other hand, for WKGCH2, the treatment with the highest growth was at 136.57 rpm and 22.93°C, showing an increase of 41.17 g with a yield of 123%. Both performances are similar, and it can be related to the increase in agitation in the case of the experiments at 250 mL, the growth increases. Laureys *et al.*, (2017) found a 20% to 50% increase in a medium composed of unrefined sugar and dried fruit at 96 hours and 21°C. The same authors, in their 2018 study under similar conditions, found yields between 38.4% and 10.6%. Comparing the results of the present study with those reported by Laureys, it can be said that the operating conditions found in this research (at 250 and 500 mL) increase the likelihood of obtaining better yields of WKG, or this may also be due to the composition and nutrient richness of the NCS.

In this research, we can observe that agitation has a greater effect when the grain size is small, since the neck diameter of the 250 mL flask used in this study is 4.3 cm, which represents a limited space, since these grains can float and remain clustered in the neck of the flask, hindering proper substrate exchange. Consequently, the differences in size and shape between the grains from Chile and Colombia are significant, with WKGCOL3 growing from 6 mm to 3.1 cm, while WKGCH2 does not exceed 5.2 mm. This is important because grain size is directly related to agitation, as smaller grains, despite their head

diameter, could move freely in the flask, causing the "grain size" condition to generate disturbances and consequently differences in results between each group of grains.

4.6.3. Operating Conditions for Lactic Acid (LA) formation

According to the results found in the ANOVA table, the fitted polynomial for LA with WKGCOL3 was of the linear type, with an R^2 value of 0.78, indicating a high correlation between the factors and the response. This may indicate that the polynomial can be used for predictive purposes.

For WKGCH2, a linear polynomial was fitted with an R^2 of 0.82, indicating that the equation explains the relationship between the factors and the responses. According to Table 4.15 for WGKCOL3, experiment 3 (136.57 rpm and 37.07°C) showed a maximum production of 11.34 g/L lactic acid and a minimum production of 1.24 g/L for experiment 8 (80 rpm and 20°C). On the other hand, for WGKCH2, as shown in Table 4.16, the maximum amount of this metabolite produced was 11 g/L under the conditions evaluated in experiment 3 and a minimum of 1.19 g/L in experiment 8. It is observed that the amounts of this metabolite decrease at lower temperatures, which is consistent with what has been described for both grains. The lowest and highest values of LA correspond to experiments 3 and 8, where the highest amount of LA was observed at a temperature of 37°C and a stirring speed of 136.57 rpm. It is known that the optimal temperature for *Lactobacilli* is defined within a range of 28 to 42°C or even higher temperatures (Jena *et al.*, 2017), which explains why these conditions favor the formation of this metabolite.

4.6.4. Operating Conditions for Ethanol (EtOH) formation

The polynomial regression found in the WKGCOL3 and WKGCH2 fermentations was of quadratic order. The polynomial for WKGCOL3 has significant terms, indicating that it can be used for future predictions. The correlation coefficient R^2 was 0.94, indicating a strong relationship between the response variable and the factors. For WKGCH2, the R^2 was 0.96 and there is no significant variation among the data.

The EtOH levels for the WKGCOL3 fermentation varied between 20.42 g/L for experiment 2 (160 rpm and 30°C) and 9.21 g/L for experiment 8 (80 rpm and 20°C), showing a significant difference between the amounts of ethanol and the treatments in question. For WKGCH2, the amounts ranged from 19.57

g/L for experiment 2 (160 rpm and 30°C) to 7.62 g/L for experiment 8 (80 rpm and 20°C); these results can be seen in Tables 4.15 and 4.16. Both grain fermentations coincide in the maximum and minimum EtOH levels in Experiments 2 and 8, which may indicate that agitation and temperature have a significant effect on ethanol production.

Amounts of ethanol between 2.14 and 4.35 g/L have been reported in an aerobic process. (Laureys y De Vuyst, 2017b). AAB are also known to grow in ethanol-rich environments under aerobic conditions. (Mohd Azhar *et al.,* 2017). Accordingly, it can be said that under the fermentation conditions of the present study, ethanol formation was favored, which is directly related to the optimal temperature range for yeasts, which is between 25 and 30°C. (Gientka *et al.,* 2017). This suggests that lowering the temperature significantly reduces the formation of ethanol, which could be a factor to consider when producing beverages aimed at audiences that do not consume alcoholic beverages and/or children who are looking for healthy foods.

The ethanol content in this type of beverage has also been reported to be between 11 and 20 g/L (David Laureys and De Vuyst, 2014b; Martínez-Torres *et al.,* 2017), indicating that WKG fermentations are alcoholic beverages that can contain significant amounts of EtOH, as was the case in the present study. It is known that functional beverages with significant alcohol content are undesirable, which is why in many countries the alcohol content must be below 0.5%, although this can vary between countries. (Bellut and Arendt, 2019). Therefore, in this experiment, conditions must be created that allow the minimum EtOH content to be reached, perhaps by operating at temperatures close to 20°C and with agitation. It is known that kefir grains are microbial consortia that represent symbiotic relationships between bacteria and yeasts, relationships that affect both the growth of the grains and the production of EtOH and LA. (Leroi and Pidoux, 1993) (Stadie, Gulitz, Matthias A. Ehrmann, *et al.,* 2013) Therefore, the production of EtOH depends not only on exogenous factors such as agitation and temperature, but also on conditions that allow the survival of bacteria that promote the development of yeasts and, consequently, their metabolism. It is necessary to understand how the operating conditions can affect each microbial group and thus direct their activity towards what is considered desirable in terms of the final fermentation product, which was analyzed or in the subsequent study, also included in this thesis, related to the metabarcoding of both grains.

4.6.5. Operating Conditions for the Formation of Acetic Acid (AA)

Acetic Acid Bacteria (AAB) are known to convert ethanol to acetic acid and are responsible for the spoilage of alcoholic beverages such as wine. (Drysdale and Fleet, 1988). Some authors have reported the presence of AAB in water kefir grains. (D. Laureys y De Vuyst, 2014; Fiorda *et al.,* 2017a; Breig y Luti, 2021a). For the purposes of this study, a fermentation setup was used to induce anaerobiosis in the system and prevent the development of AAB, where 250 mL flasks were filled to the usable volume, leaving a free space of approximately 50 mL to promote anaerobiosis. Although oxygen is initially present in the headspace of the flask, the consumption of oxygen tends to be relatively rapid because the microorganisms that initiate fermentation have a high rate of carbon dioxide production early in the fermentation process, which quickly removes a significant portion of the oxygen present. (Laureys *et al.,* 2018). As a result, in this experiment, it was observed that the anaerobic atmosphere was effective in preventing the development of AAB in water kefir-based beverages.

For WKGCOL3, quadratic order models were fitted with an R^2 of 0.79. According to these parameters, this polynomial can be used for prediction purposes. On the other hand, for WKGCH2, a quadratic polynomial was also fitted with an R^2 of 0.91, indicating a very good relationship between the response variable and the factors. This suggests that the empirical model obtained for WKGCH2 can be used to predict the fermentations of WKG, since the results for both grains are relatively similar. In addition, in this experiment, 250 mL gave the best fits for the models compared to the 500 mL experiment, which helped in obtaining extrapolatable equations to analyze this type of fermentation.

According to the data found in Table 4.15, for this variable in WKGCOL3, the maximum value of AA was 1.01 g/L in experiment 9 (80 rpm and 30°C) and the minimum value was 0.26 g/L in treatment 8 (80 rpm and 20°C). For WKGCH2, the maximum value of AA was 0.62 g/L in experiment 2 (160 rpm and 30°C) and the minimum value was 0.22 g/L in experiment 8 (80 rpm and 20°C). As shown in Table 4.16, these data indicate that the amount of this acid decreases at the lowest temperature used in this design (20°C). It can also be concluded that the amount of AA in both fermentations is minimal in all treatments. The acetic acid content in water kefir fermentation, along with the production of lactic acid, causes a significant decrease in pH from 5.0-6.0 to 3.4-3.6 (unpublished data), which can cause the grains to stop growing or even die (Laureys *et al.,* 2019). Reports have indicated acetic acid levels in this fermentation ranging from 0.4 g/L to 1.4 g/L (Karina Teixeira Magalhães *et al.,* 2010), which is very similar to that obtained in this study. Furthermore, the optimal fermentation temperature for this microbial group is around 30°C, which suggests that their activity at temperatures outside this range may

inhibit the growth of AAB, which is consistent with the results of this study, since the highest levels of this organic acid were found at 30°C.

To analyze how the microbial groups of water kefir (BAL, AAB, and LEV) can intervene in the development of flavor (represented in terms of the main metabolites LA, AA, and EtOH), a comparative table is shown in the Appendix 8.5, Table F. For lactic acid flavor, it was found that at condition 3 (37°C), lactic acid is found in its highest proportion, with a value of 11.34 g/l, but LAB, on the other hand, shows its minimum at the same condition. For the same metabolite, it was found that the minimum values of LA (1.2 g/L) and the maximum values of BAL occurred at the lowest temperatures of 20 and 23 °C, respectively. This allows us to conclude that at temperatures between 20 and 23 °C, the lactic acid bacteria will express more, and the LA will be outside the desired ranges of the regulations and of the taste profile initially established in the methodological strategy. It is also concluded that temperatures between 37 and 30 °C favor the production of lactic acid in the desired range and the formation of lactic acid bacteria cells.

On the other hand, for the development of EtOH taste in the beverage, where a lower percentage (very minimal) of 1% is desired, it was found that at 20°C (condition 8) the formation of ethanol was 0.7%, which would be desirable for the development of a functional beverage. The maximum content of the same metabolite occurred between 30 and 37°C with values up to 2.5%. Yeasts, the main producers of this EtOH, express their maximum abundance also at temperatures close to 23°C (condition 1), which indicates (as for LAB and its main metabolite - LA) that the metabolism of LEVs is oriented towards the formation of ethanol at temperatures close to 30°C and towards the formation of new microbial cells at temperatures close to 20°C.

Finally, for acetic acid, it was found that this metabolite was formed to a lesser extent at 20°C, relating its low formation to the low EtOH production. AAB showed a minimum at temperatures below 30°C, indicating that temperatures between 20 and 30°C, are unfavorable for the formation of acetic acid and therefore for the development of the flavor of the fermented beverage. At temperatures around 30°C, a sensorially pleasing beverage can be guaranteed.

4.6.6. Operating Conditions for Sugar Consumption

Sucrose is the most important component in the composition of panela (65-85%), followed by the reducing sugars GLUC and FRUC (10-15%) (Guerra and Mujica, 2010; Walter R. Jaffé, 2015; Breig and Luti, 2021b). In this experiment, an 8% panela was used as substrate, obtained from a factory with a declared composition of 93% carbohydrates. According to the data obtained in this experiment, the GLUC content for the fermentation of WKGCOL3 varied from 3.83 to 10.70 g/L (treatments 2 and 8), the FRUC varied from 4.99 to 19.67 g/L (treatments 2 and 4), and the SUCR varied from 8.16 to 35.72 g/L. (treatments 3 and 8). In the case of WKGCH2, sugar contents varied from 4.97 to 11.97 g/L (treatments 2 and 8) for GLUC, from 6.43 to 23.40 g/L (treatments 2 and 4) for FRUC, and from 7.97 to 38.95 g/L (treatments 4 and 8) for SUCR. Tables 4.15 and 4.16 show the amounts of GLUC, FRUC and SUCR at the end of fermentation and in each treatment, highlighting a very similar behavior for both grains in terms of sugar content. This shows trends and similarities that indicate a strong possibility that both grains have a very similar microbial composition and a common ancestral grain of origin that adapted to each substrate in the different regions of Latin America.

For GLUC, quadratic order empirical models were found in both grains. In WKGCOL3, the polynomial had an R^2 of 0.93, indicating that this empirical model can be used for prediction according to these parameters. On the other hand, for WKGCH2, GLUC was fitted to a quadratic polynomial with an R^2 of 0.86, indicating a good relation between the response variable and the factors. In the case of FRUC, quadratic models were found for WKGCOL3 with an R^2 of 0.87, indicating that the model theoretically has no significant deviations. FRUC (WKGCH2) also presented a significant quadratic polynomial with an R^2 of 0.86, indicating that this model can correlate the response with the factors without significant deviations. Regarding the results obtained for SUCR, it was observed that quadratic models were fitted for fermentation in both grains. In the case of WKGCOL3, the model was significant with an R^2 of 0.95, indicating that the response variables may be fully represented by the polynomial found. For SUCR in WKGCH2, a second order model was found with a p-value <0.0001, and the lack of fit was significant with a p-value <0.0005, which may suggest changing the model or reducing the terms. The recommended transformation (base 10 logarithm) was performed, and the new fitted and predicted R^2 values were closer at 0.95 and 0.93, respectively, indicating that the transformation of the data provided a better fit to the polynomial. These two measures overcame this specific problem, and according to the literature, this method allowed for the evaluation of the explanatory power of the regression model. (Minitab Blog Editor 2019).

As reported by Teixeira *et al.*, in their 2010 study, the content of reducing sugars increases after 24 hours of fermentation due to hydrolysis of sucrose. In this study, at 96 hours, the maximum FRUC content in WKGCH2 increased only in experiment 8 (80 20°C), just as for FRUC, which also increased but to a lesser extent in the same experiment for both grains. This may indicate the presence of some microbial groups, as in the case of the 500 mL experiment. Laureys *et al.*, in 2018 show that after 72 hours of fermentation, fructose levels increased in anaerobic fermentations carried out with the substrate commonly used in Europe (sucrose with dried figs), which also indicates the hydrolysis of sucrose and, therefore, the increase of some reducing sugars. As can be seen, the results for both grains showed similar trends in sugar consumption, so the models found in this design can be used for predictive purposes in WKGs of other origins.

In Appendix 8.5, Table G, the trends in sugar consumption and/or similarities between the experiments performed (250 and 500 mL) can be observed, with the aim of observing the reproducibility of the models found and the consistency of the data, as well as to see in the final fermented beverages if the sugar content is within the desired flavor and regulatory profile.

For glucose, it was generally observed in all experiments that the maximum consumption was obtained at temperatures between 30 and 37 °C and the lowest at 20 °C. For fructose, it was found that the highest consumption occurred at temperatures of 30°C and the lowest at 40°C, and finally for sucrose, it was found that the highest consumption occurred at temperatures above 37°C, contrary to the other sugars, indicating that when sucrose is hydrolyzed, it is very likely that at this temperature the other sugars are not consumed by the microorganisms. The latter is confirmed by the fact that the lowest total amounts were found at temperatures below 30°C. Finally, as far as the taste profile is concerned, at temperatures below 30°C, fermented beverages can be obtained that meet the current standard for healthy foods with low sugar content.

4.6.7. Regressing Polynomials obtained by RSM

Table 4.17 shows the summary of the coefficients for the polynomials found for all response variables in relation to the factors. The equations and results found could be used to predict and search for desired results. For numerical optimization, limits were considered based on the logic of the experiment itself and what was considered desirable. The range means that the variable can move between any of the limits, while the importance is the weight assigned to each response variable.

In WKGOL3, linear models were found for the response variables LA and FG, while quadratic polynomials were found for the remaining variables. In the case of WKGCH2, it was found that, as in WKGCOL3, a linear empirical model was fitted for LA and quadratic order polynomials for the other response variables. The Table C (Appendix 8.5), describes the importance of the response on a scale of 1 to 5. These constraints are presented as ranges, defined with the goal of finding operating points that meet the desired conditions.

4.6.8. Prediction point found for fermentation operating conditions

Table 4.18 shows the model fits for WKGCOL3 using restrictive measures (targets). It also shows the prediction point that was found to be the best solution in terms of desirability, calculated as a function of the Global Desirability (GD), which was 0.588. The DG confirms that the operating point found was very close to the predicted desirability value, with the operating point being agitation (57.14 rpm) and temperature (37.07°C).

Table 4.17. *The empirical models obtained for each response variable in each grain are presented in terms of factors (X1 rpm, X2 °C) and regression coefficients bi. The order of each equation is determined by the degree of the factors; for both grains, first and second-degree polynomial equations were presented.*

	WKGCH2				**Full models**			
Factor	**Regression Coefficient**	**Final grain (FG) g/L**	**Lactic acid (LA) g/L**	**Acetic acid (AA) g/L**	**Ethanol (EtOH) g/L**	**Glucose (GLUC) g/L**	**Fructose (FRUC) g/L**	**Sucrose (SUCR) g/L**
	Linear							
	b_0	141.8113	-9.17662	-0.881817	-23.61635	27.81694	44.56363	73,3110
X_1 - RPM	b_1	0.007678	-0.010321	-0.003421	-0.115539	0.082605	0.008912	0,2164
X_2 -T	b_2	-1.29081	0.450649	0.085724	2.43231	-1.41213	-2.34112	-2,8985
	Quadratic							
X_1*X_2	$b3$			-0.000025	0.000119	0.000038	0.002519	-0,0031
X_1^2	$b4$			0.000028	0.000779	-0.000544	-0.000693	-0,0007
X_2^2	$b5$			-0.00128	-0.034811	0.021269	0.040587	0,0305
	WKGCOL3				**Full models**			
Factor	**Regression Coefficient**	**Final grain (FG) g/L**	**Lactic acid (LA) g/L**	**Acetic acid (AA) g/L**	**Ethanol (EtOH) g/L**	**Glucose (GLUC) g/L**	**Fructose (FRUC) g/L**	**Sucrose (SUCR) g/L**
	Linear							
	b_0	115.5511	-8.073	-2.97102	-8.92472	28.35188	46.3382	70,31061
X_1 - RPM	b_1	-0.259696	0.011678	0.000739	-0.121362	0.076894	0.010339	0,21324
X_2 -T	b_2	1.13699	0.402029	0.240792	1.64515	-1.52514	-2.60933	-2,95326
	Quadratic							
X_1*X_2	$b3$	0.001435		0.000044	-0.000175	-0.000113	0.002544	-0,00271
X_1^2	$b4$	0.001516		-4.75236E-06	0.000845	-0.000484	-0.000664	-0,00083
X_2^2	$b5$	-0.037026		-0.004005	-0.022878	0.023403	0.044247	0,03483

X_1 = Agitation; X_2 = Temperature

Table 4.18 *The general prediction point (level) for optimization can be observed for WKGCOL3, the standard deviation, the coding of the factors, and the minimum and maximum values considered.*

Factor	Name	Level	Low Level	High Level	Std. Dev.
X_1	**Agitation (rpm)**	57.14	23.43	136.57	0
X_2	**Temperature (°C)**	37.07	22.93	37.07	0

Table 4.19 shows the expected average predicted values for each response, the standard deviation, and the confidence interval within which the predictions of the empirical model can vary.

Table 4.19. *Possible solutions for each response variable according to the empirical models constructed, the standard deviation and its confidence interval, for WKGCOL3.*

Solution Response (g/L)	Predicted Mean	Std Dev	95% CI low for Mean	95% CI high for Mean
FG	99.968	3.90707	95.0212	104.915
AL	7.49749	1.31906	6.14556	8.84942
AA	0.570442	0.138171	0.395501	0.745382
EtOH	16.076	0.864199	14.9819	17.1702
GLU	6.55133	0.703904	5.66011	7.44256
FRU	14.224	1.33284	12.5364	15.9115
SUCR	10.1749	3.20812	6.88685	13.463

Figure 4.12 shows, in the form of a ramp chart, the values set as operating conditions and the expected values for each response according to the selected conditions. The red points indicate the position in the sample space for the factors, while the blue points indicate the expected results for the response variables in g/L. The ramps are sloped according to the trend (maximizing or minimizing the result) and the points move according to the quantities expressed in the established ranges. For WKGCH2, the restrictions applied, the variable limits, and the importance or weight assigned to certain variables are shown in Table C (Appendix 8.5).

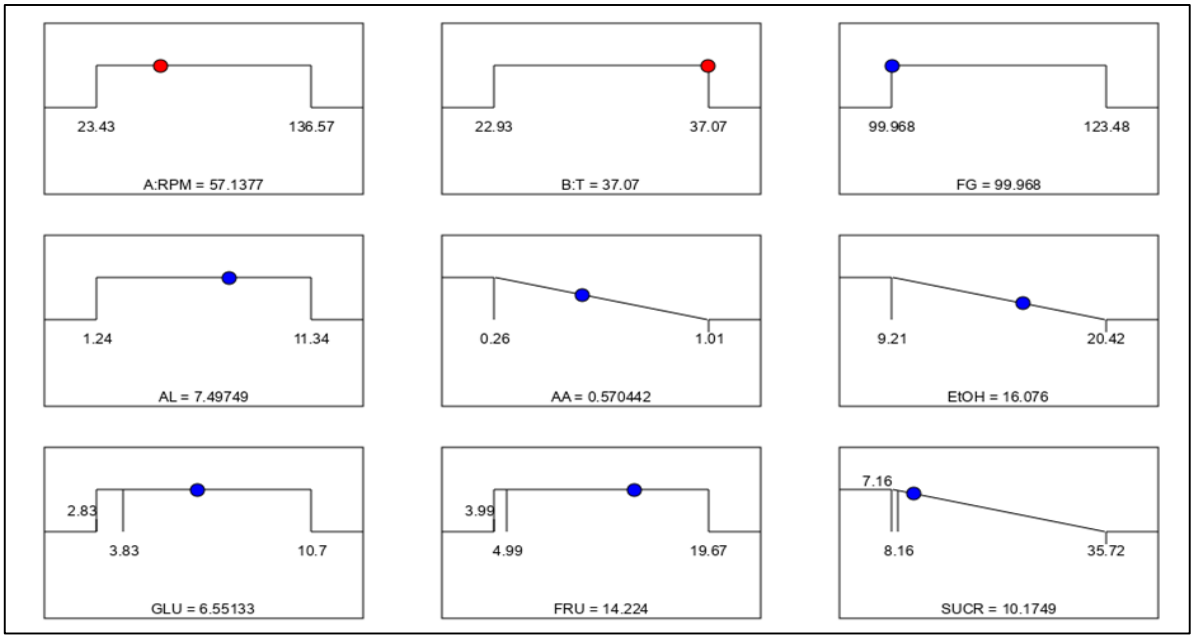

Figure 4.12. *Numerical solution for WKGCOL3 observed in ramp plots according to the constraints used. The values at the ends of the ramps are the limits used to decrease or increase the sample range; the value at the bottom of each ramp corresponds to the predicted value for each response variable. The red dots represent the factors, and the blue dots represent the responses.*

Table 4.17 shows the fitted polynomials for predicting the responses of grain WKGCH2, using the same restrictive measures and bounds implemented for the WKGCOL3 experiment. Table 4.20 shows the prediction point for WKGCH2 that was found to be the best solution according to the desirability function, calculated as Global Desirability (GD), which was 0,688. The GD confirms that the operating point found is very close to the predicted desired value. The expected operating point found for WKGCH2 is Agitation (102.99 rpm) and Temperature (37.07°C).

Table 4.20. *The general prediction point (level) for optimization can be observed for WKGCH2, the standard deviation, the coding of the factors, and the minimum and maximum values considered.*

Factor	Name	Level	Low Level	High Level	Std. Dev.
A	**Agitation (rpm)**	102.99	23.43	136.57	0
B	**Temperature (°C)**	37.07	22.93	37.07	0

Depending on the polynomial chosen and the constraints used, Table 4.21 shows the expected values of each response for WKGCH2, the standard deviation, and the confidence interval within which the model's predictions can vary. Figure 4.13 shows the numerical prediction points that meet the given constraints. The prediction point found was agitation (102.94 rpm) and temperature (37.07°C). The numerical solution is shown in ramp plots (For WKGCH2).

Table 4.21. *Possible solutions for each response variable according to the empirical models constructed, the standard deviation and its confidence interval, for WKGCH2.*

Solution Response (g/L)	Predicted Mean	Std Dev	95% CI low for Mean	95% CI high for Mean
FG	93.1704	5.44106	87.5919	98.749
AL	8.59148	1.3047	7.25381	9.92915
AA	0.383219	0.0381721	0.334863	0.431575
EtOH	15.5263	0.769304	14.5518	16.5009
GLU	7.58362	0.924107	6.41297	8.75427
FRU	16.7357	2.01169	14.1873	19.2841
SUCR	11.041	2.06626	8.42352	13.6586

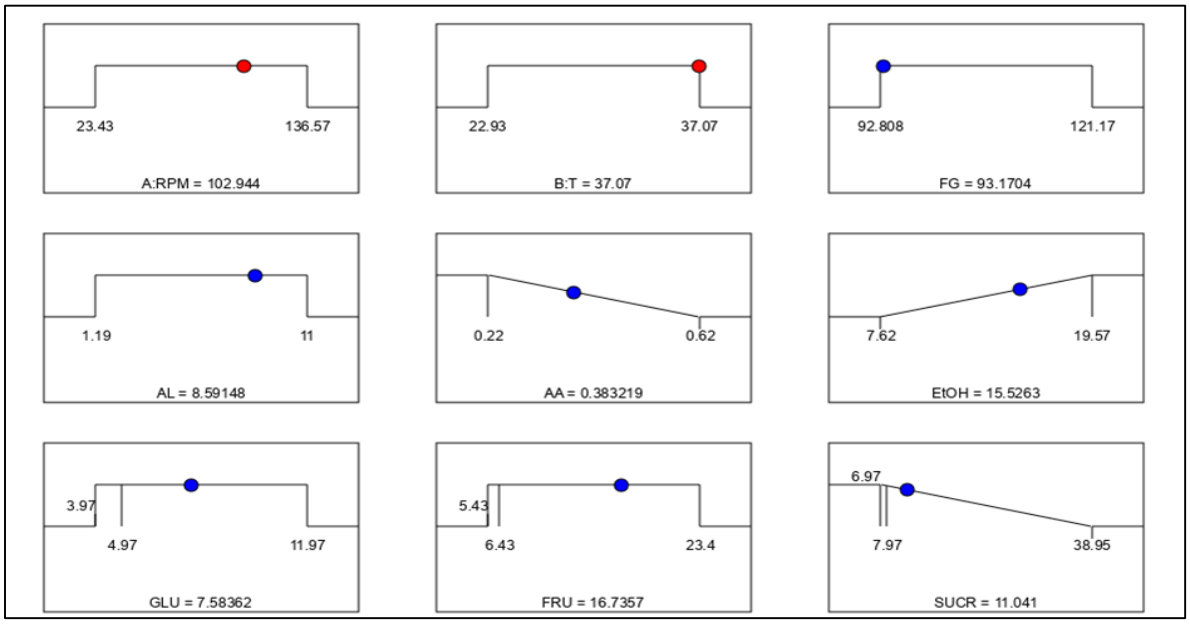

Figure 4.13. *Numerical solution for WKGCH2 observed in ramp plots according to the constraints used. The values at the ends of the ramps are the limits used to decrease or increase the sample range; the value at the bottom of each ramp corresponds to the predicted value for each response variable. The red dots represent the factors, and the blue dots represent the responses.*

A general optimization was also carried out for both grains using the same constraint criteria described (Table C, Appendix 8.5), and it was found that the operating point coincides at a temperature of 37°C, just as in the experiment carried out at 500 mL and a stirring speed of 79 rpm, with an acceptable DG of 0.609. (Figure 4.14). Therefore, the operating point to obtain a standard beverage with any of the kefir grains studied, at volumes of 250 and 500 mL and with the desired characteristics according to the contents of ethanol, acids, sugars and biomass increase, was established at 37°C with agitation between 38 and 79 rpm (Table 4.22). Considering that a large volume of data was worked with, and the quantification techniques are of high resolution, it can be stated that the models can be used for prediction purposes in other processes with water kefir grains that estimate similar characteristics regarding the imposed environmental conditions. Table 4.23 shows the expected predicted means for each response in global optimization. It is interesting to observe how the design was able to establish equal and/or similar

conditions for two grains of different origin and at two volume scales, confirming that Response Surface Designs (RSM) are very useful for finding optimal operating conditions in biological processes due to their great capacity for statistical analysis through various indicators of goodness of fit and data dispersion. The beverages obtained from the fermentation of NCS with water kefir grains of Latin American origin were standardized to process conditions that favor optimal properties for the development of flavor and the growth of biomass and microbial groups with probiotic potential.

Table 4.22. *The general prediction point (level) for optimization can be observed with both grains (WKGCOL3 and WKGCH2), the standard deviation, the coding of the factors and the minimum and maximum values.*

Factor	Name	Level	Low Level	High Level	Std. Dev.
X_1	**Agitation (rpm)**	79,20	23.43	136.57	0.000
X_2	**Temperature (°C)**	37,07	22.93	37.07	0.000

Table 4.23. *Possible solutions for each response variable according to the models constructed for with both grains (WKGCOL3 and WKGCH2), the standard deviation, and its confidence interval.*

Solution Response	Predicted Mean	Std Dev	95% CI low for Mean	95% CI high for Mean
		WKGCOL3		
FG	99,97001374	1,993941033	95,37197751	104,56805
LA	7,755194465	0,584645046	6,468399397	9,041989534
AA	0,608252509	0,070515908	0,445642535	0,770862483
EtOH	15,79693782	0,441047195	14,77988118	16,81399447
GLUC	6,701019322	0,359240048	5,872610293	7,529428351
FRUC	14,53459431	0,680219612	12,96600508	16,10318353
SUCR	12,43799294	0,961646381	10,22043243	14,65555345
		WKGCH2		
FG	93,3537017	2,411656808	88,04568087	98,66172254
LA	8,34644523	0,578282186	7,073654723	9,619235737
AA	0,366805624	0,019481278	0,321881718	0,411729531
EtOH	14,7967251	0,392617382	13,8913478	15,7021024
GLUC	7,939892702	0,471621774	6,852330951	9,027454452
FRUC	17,30535697	1,026675907	14,93783811	19,67287584
SUCR	11,56970747	1,054522589	9,137974042	14,0014409

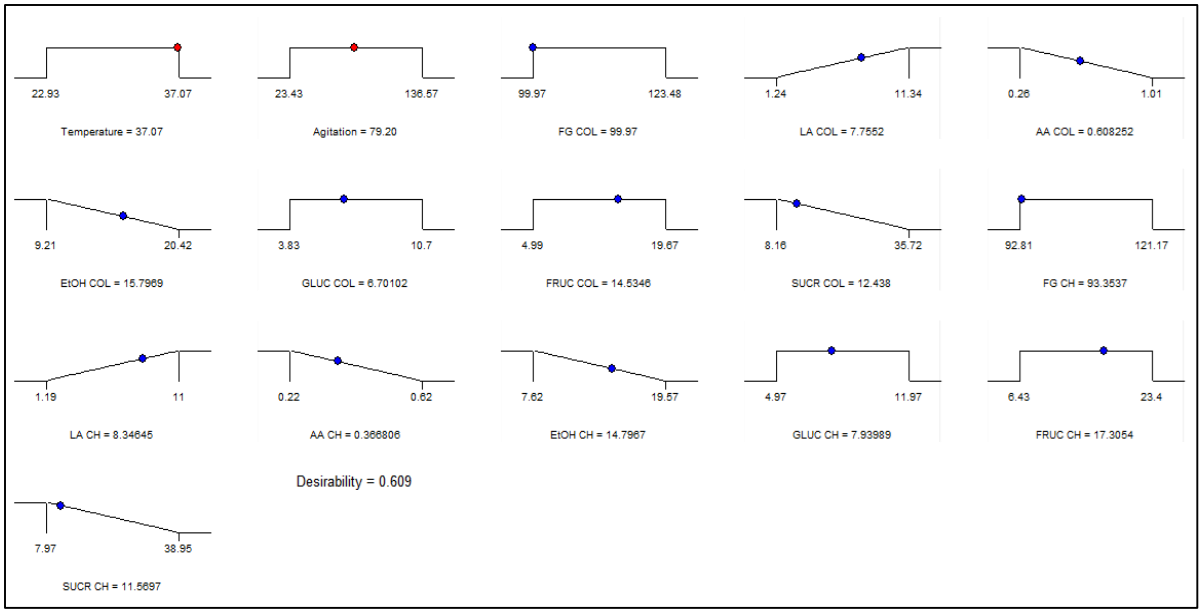

Figure 4.14. *Numerical solutions for both grains (WKGCOL3 and WKGCH2) observed in ramp plots according to the constraints used. The values at the ends of the ramps are the limits used to decrease or increase the sample range; the value at the bottom of each ramp corresponds to the predicted value for each response variable. The red dots represent the factors, and the blue dots represent the responses.*

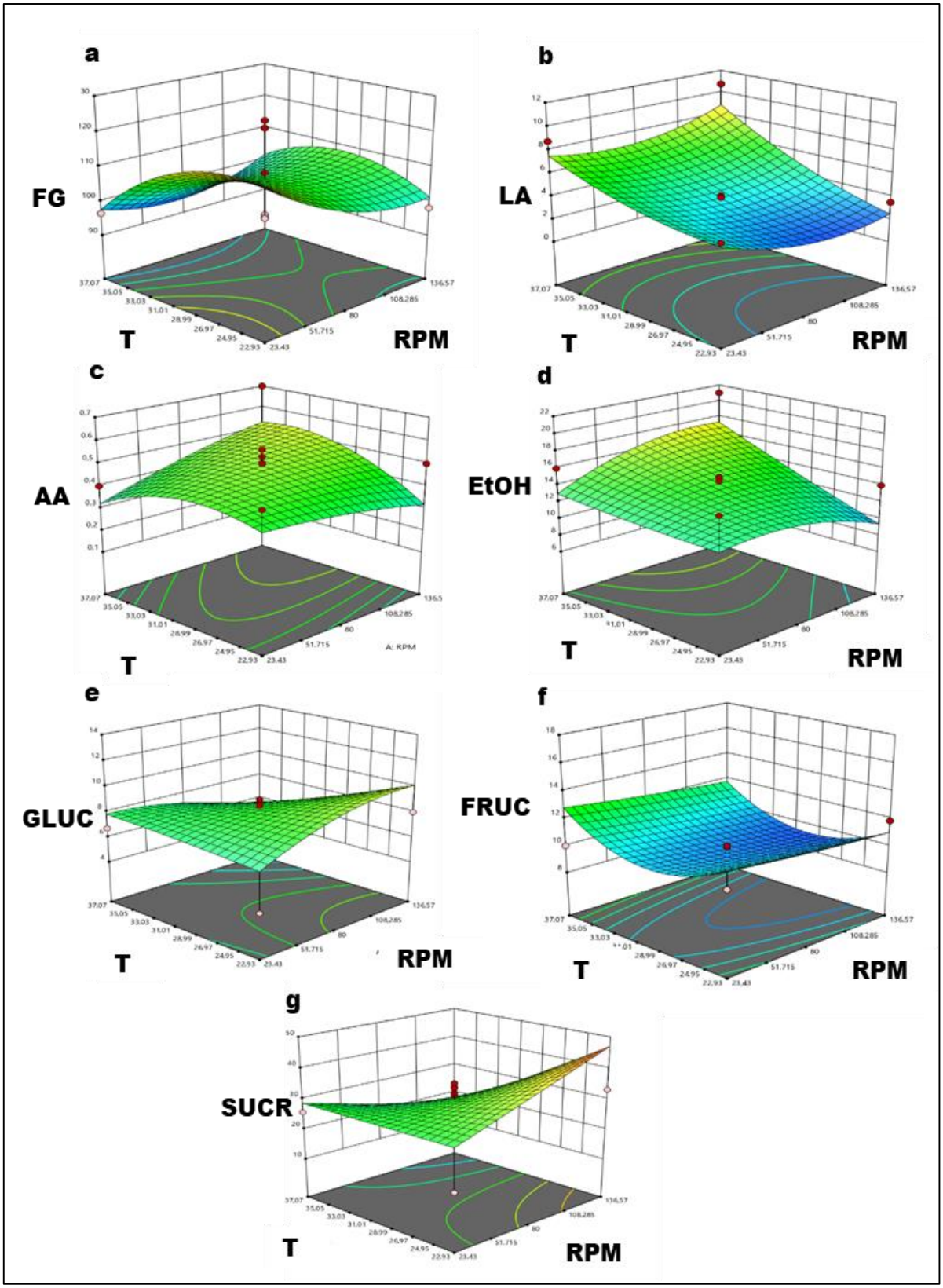

Figure 4.15. *Chilean water kefir response surface plots (3D), experiment at 250 mL. The effects of Temperature (T) and Agitation (RPM) on (a) Final grain - FG; (b) Lactic acid - LA; (c) Acetic acid AA; (d) Ethanol - EtOH; (e) Glucose - GLU; (f) Fructose – FRU; (g) Sucrose – SUC. All results for variables are reported in g/L.*

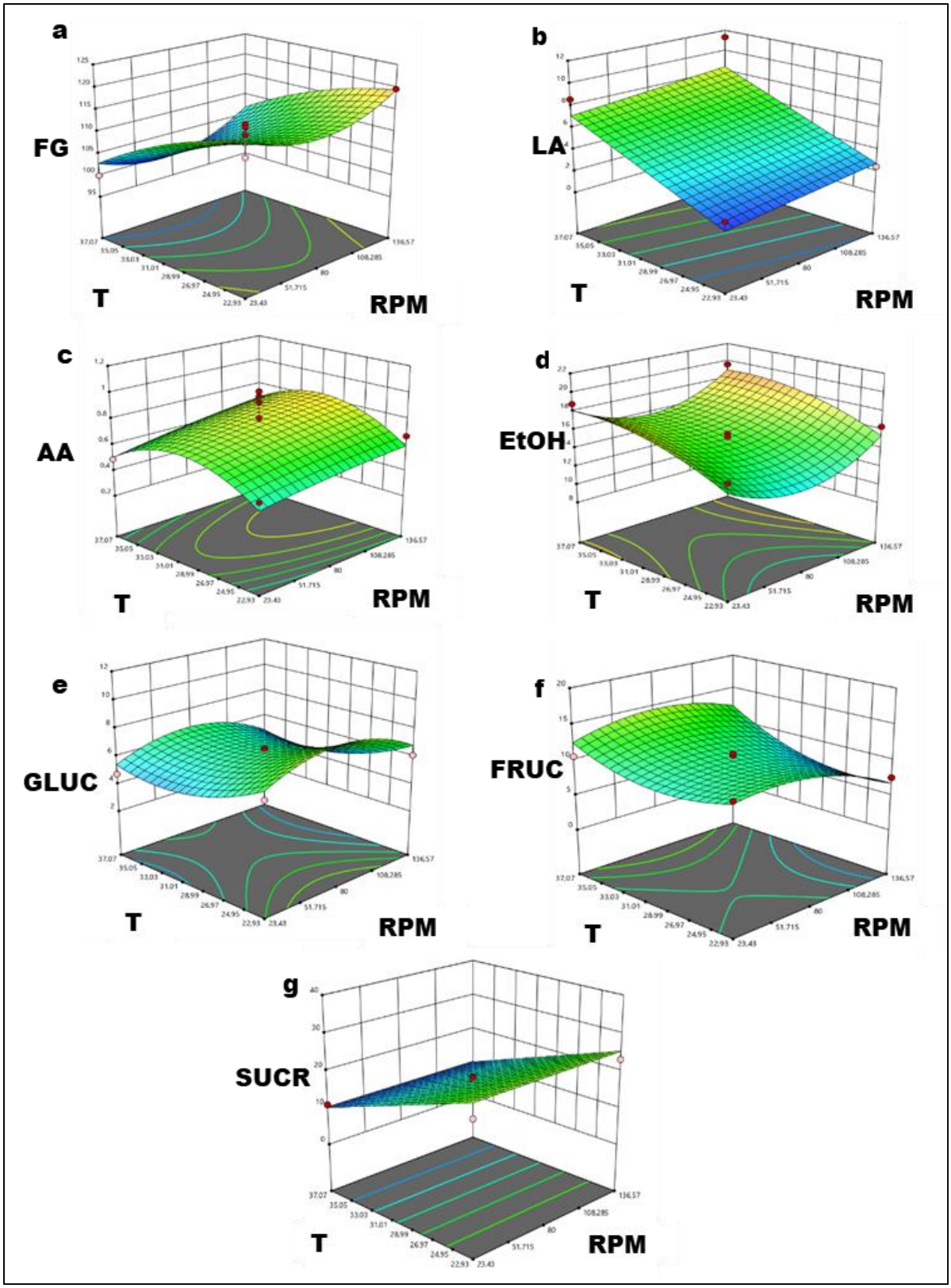

***Figure 4.16**. Colombian water kefir Response surface plots (3D), experiment at 250 mL. The effects of Temperature (T) and Agitation (RPM) on (a) Final grain - FG; (b) Lactic acid - LA; (c) Acetic acid - AA; (d) Ethanol - EtOH; (e) Glucose - GLU; (f) Fructose – FRU; (g) Sucrose – SUCR.*

4.7. Metabarcoding Optimization Study of Kefir Grains

Since this metabarcoding analysis was carried out under the same central composite design (CCD; see Methodology Section in Table 3.3) used for the beverage standardization studies, RSM was also used to find operating points that included the abundance of major microbial groups and thus be able to make correlations with the formation of metabolites (metabolites that determine the sensory qualities of the fermented beverage) and biomass with the microorganisms present. In addition, changes were analyzed by calculating the standard deviations (SD) of the main microbial groups and species in response to modifications caused by variations in temperature and agitation, to identify patterns that would allow a deeper understanding of the fermentation process. The results of the optimization considering the abundance of the main microbial groups are shown in Table 4.24. The results, including all microbial genera can be seen in Appendix 8.6, Tables A and B.

Table 4.24. The results generated in the CCD used for optimization are shown in this table. The abundance of Lactobacilli, Acetic Acid Bacteria and Yeasts is expressed in number of annotated reads, while the main metabolites (LA, AA and EtOH) and the growth of the grains are expressed in g/L.

WKGCH2								
Temperature	**Agitation**	**Lactobacillus**	**Acetobacteria**	**Yeast**	**FG**	**LA**	**AA**	**EtOH**
°C	rpm	Abundance	Abundance	Abundance	g/L	g/L	g/L	g/L
30,00	80,00	38022	81	17024	100,064	4,91	0,41	14,05
30,00	160,00	39425	59	15002	102,25	6,15	0,62	19,57
20,00	80,00	54425	252	33519	106,96	1,19	0,22	7,62
37,07	23,43	51150	224	27506	92,81	10,21	0,49	17,48
22,93	23,43	62942	81	33753	120,26	1,98	0,33	11,25
30,00	80,00	36778	112	16885	98,88	4,83	0,40	14,00
22,93	136,57	55159	247	39161	121,17	2,17	0,33	12,75
40,00	80,00	34127	484	24559	93,84	7,15	0,30	12,80
30,00	0,00	34943	95	21282	106,31	3,54	0,51	17,78
37,07	136,57	33355	169	17936	94,17	11.00	0,45	19,17
30,00	80,00	34718	134	16707	99,54	4,78	0,34	14,15
30,00	80,00	37887	125	16889	99,80	4,84	0,37	14,10
30,00	80,00	35698	111	17001	98,62	4,86	0,39	14,20
30,00	80,00	36545	115	16995	99,95	4,75	0,38	13,80
WKGCOL3								
Temperature	**Agitation**	***Lactobacillus***	***Acetobacteria***	**Yeast**	**FG**	**LA**	**AA**	**EtOH**
°C	rpm	Abundance	Abundance	Abundance	g/L	g/L	g/L	g/L
30,00	80,00	40032	123	15522	111,87	4,75	0,63	14,93
30,00	160,00	42114	147	10866	123,48	4,89	0,81	20,42
20,00	80,00	45765	120	19988	112,24	1,24	0,26	9,21
37,07	23,43	46058	44	22637	99,97	8,58	0,49	18,78
22,93	23,43	53865	220	23179	120,2	2,2	0,54	15,29
30,00	80,00	45765	120	19988	108,22	4,87	0,7	15,62
22,93	136,57	69067	103	55426	119,62	2,42	0,67	16,36
40,00	80,00	39404	303	27890	100,44	6,5	0,44	14,81
30,00	0,00	49589	112	21287	116,00	3,26	0,63	18,99
37,07	136,57	39702	102	18888	119,62	2,42	0,67	16,36
30,00	80,00	41258	118	25502	111,144	4,78	1,01	15,25
30,00	80,00	47153	125	17989	107,54	4,67	0,61	14,86
30,00	80,00	49579	100	20145	109,342	4,72	0,81	15,05
30,00	80,00	48383	140	20998	104,12	4,69	0,93	15,00

In general, the dominance of the *Lactobacillus* genus was observed and reconfirmed in the grains of this study; for WKGCOL3, the highest abundance for this microbial genus was 69,067 in experiment 1 (23 °C, 136 rpm) and the lowest was 39,404 in experiment 4 (40 °C, 80 rpm). For WKGCH2, the highest abundance was also 62,942 in experiment 7 (23 °C, 23 rpm), while the lowest abundance was found in experiment 3 (37 °C, 137 rpm). While *Lactobacilli* are known to grow at temperatures between 30 and 40 °C (Śliżewska *et al.*, 2020), in this microbial community their expression was better represented at 20 and 23 °C in both grains. Regarding the formation of LA as the main metabolite produced by *Lactobacilli*, it was found that the highest LA contents were observed at 37°C, while at 23°C both grains yielded acid contents between 2 and 2.4 g/L.

In the case of biomass, the highest growth was recorded in both grains during experiment 7 (23°C, 23 rpm). The above indicates that the abundance of *Lactobacillus* is directly related to the growth of grains (dextran-producing *lactobacilli*) and that the formation of LA as a metabolite mainly produced by LAB is conditioned by the enzymatic machinery that is mainly activated at 37°C, possibly directing its central metabolism towards lactate formation rather than biogenesis.

Regarding the acetic acid bacteria, it was found that the highest abundance in both grains occurred under condition 4 (40°C, 80 rpm) with abundances of 484 and 303 (for WKGCH2 and WKGCOL3, respectively), and the lowest abundance was observed under condition 2 (30°C, 160 rpm) for grain WKGCH2 with an abundance value of 59, while for WKGCOL3 a count of 44 was found under condition 5 (37°C, 23 rpm). Acetic acid (AA) was formed at a higher rate for both grains under condition 2 (30°C, 160 rpm), with values of 0.62 g/L and 1.01 g/L for WKGCH2 and WKGCOL3, respectively, while it was formed at a lower rate for both grains under condition 8 (20°C, 80 rpm), with values of 0.22 and 0.26 g/L for WKGCH2 and WKGCOL3, respectively. This last point indicates a direct relationship between the higher formation of this acid at temperatures above 30°C and the lower formation at lower temperatures (20°C). There is also a direct relationship with temperature regarding the abundance of *acetobacter*, which are favored at temperatures above 37°C. However, based on these results, it is difficult to establish a direct relationship between their expression and the conditions evaluated. In this certainly complex fermentation, several factors influence the biogenesis and metabolism of these bacteria, some of which are the presence of ethanol and oxygen. Considering the criteria of this study, the aim is not to influence this group of microorganisms and to prevent the formation of AA to obtain a product with more pleasant sensory characteristics. Therefore, for optimization purposes, constraints were used to minimize the response variables AA and *Acetobacter*. It is important to understand that above 30°C, both the formation of AA and the genesis of *Acetobacter* are favored.

The yeasts showed higher abundances for both grains in condition 1 (23°C, 136 rpm) with values of 39161 and 55426 for WKGCH2 and WKGCOL3 respectively, while the lowest abundances were observed in condition 2 (30°C, 160 rpm) for both grains. This allows the identification of patterns that relate the conditions to the expression of these microorganisms, along with the operational factors. Ethanol was produced at a higher rate in condition 2 (30°C, 160 rpm) for both grains, while it was produced at a lower rate in condition 8 (20°C, 80 rpm) for both grains. For this group of microorganisms, clear relationships can be found between the evaluated conditions and the expression in abundance and EtOH. According to this information, the analysis carried out for *lactobacilli* is similar in this case, where it is observed that at temperatures above 30°C, the metabolism of the yeasts in this consortium is generally directed towards the formation of products, while at an average temperature of 23°C, biogenesis is favored.

As we know, water kefir is a complex microbial community, which in this case includes a total of 11 species of bacteria and 5 species of yeast. The same microorganisms were identified in the preliminary metabarcoding study, except for *O. kitaharae.* This optimization study validated the microbial composition of water kefir grains from Colombia and Chile. The relative abundances are detailed for each experiment in Appendix 8.6, Table A y B, determined at 48 and 96 hours for different species and genera. The most abundant bacterial species in WKGCOL3 was *L. ghanensis* with 33%, followed by *L. harbinensis* (16%), *L. casei/paracasei* (12.5%), *L. hilgardii* (12.5%), *L. parabuchneri* (9%), *L. diolivorans* (6%), *O. oeni* (4%), *O. kitaharae* (3%), *O. alcoholitolerans* (1.7%), *L. perolens* (1.3%), and *L. satsumensis* (0.02%). For the same grain, the most abundant yeast was *S. cerevisiae* with 77%, followed by *D. bruxellensis* (18%), *S. pombe* (2%), *Z. florentina* (1.5%), and *S. ludwigii* (1.5%). For WKGCH2, *L. ghanensis* was also the most abundant species with a relative abundance of 44%, followed by *L. casei/paracasei* (15%), *L. harbinensis* (12%), *L. parabuchneri* (8.4%), *L. hilgardii* (7%), *O. kitaharae* (4.8%), *L. diolivorans* (4.6%), *L. perolens* (2%), *O. oeni* (0.4%), *O. alcoholitolerans* (0.2%), and *L. satsumensis* (0.11%). Regarding the yeasts for WKGCH2, it was found that the most abundant yeast was *S. cerevisiae* with 74%, followed by *D. bruxellensis* (17%), *Z. florentina* (6%), *S. pombe* (1.4%), and *S. ludwigii* (1.3%).

Given this microbial complexity, addressing the analysis in this study, where the main objective is to find operational conditions that allow the production of fermented beverages with specific microbiological and physicochemical characteristics, it becomes necessary to process the data from a more global perspective that allows the identification of microbial behavior patterns. For the purposes of optimization, the main groups of microorganisms (*Lactobacillus*, acetic acid bacteria and yeasts) have

been considered as unique response variables, composed of different species that share metabolic characteristics that allow these groups to function together towards a primary goal.

Therefore, the optimization analysis was performed using the frequency counts annotated for *Lactobacilli*, acetic acid bacteria, and yeasts. The statistical indicators that assess the fit and accuracy correlating the responses with the operational factors provide sufficient validity to support the robustness of the results obtained by the replications at the central point, which provide a constant prediction variance at equidistant points from the center of the design. Table E (Appendix 8.6) shows the analysis of variance for the responses involving the microbial groups (lactic acid bacteria, acetic acid bacteria, and yeasts).

According to Table E (ANOVA in appendix 8.6), it is possible to determine whether the terms for evaluating the behavior of the three responses are significant or not. In the case of WKGCH2, it was found that the model fits for the three main groups are significant with p-values <0.05, indicating that these fits can be used for predictive purposes. In the case of WKGCOL3, it was found that the best fit was provided by LAB. On the other hand, when analyzing the adjustments according to the R^2 values, the correlations that fit well were those for WKGCH2 with coefficients above 0.73, and in the case of WKGCOL3, the correlation for AAB was best represented by the regression with a fit of 0.8. The Adeq. Presicion, is also an indicator of whether the model can be used in the sample space of the design; values above 4 are desirable, which also helped to determine whether the results have a good fit and their appropriate statistical validity. For all responses in both grains, except for yeasts in WKGCOL3, adequate precision values were presented to allow the use of regression adjustments in the sample space.

Based on the results presented in Table 4.25, it was determined that the optimal operating point is at 37 °C and 54 rpm. This point was determined by maximizing the LA and Temperature, minimizing the AA, AAB, YEAST, EtOH and Agitation, and keeping the FG, LAB within range. These constraints were applied according to the criteria described in Section 3.4 of the Methodology. Global Desirability (GD) was found to be at a good margin with a value of 0.629.

Table 4.25. *The general prediction point (level) for optimization, the standard deviation, and the minimum and maximum values considered for each response are detailed. The possible solutions for each response variable are also shown according to the empirical models constructed, the standard deviation and its 95% confidence interval. The responses are presented according to the use of these words: LAB (lactic acid bacteria), AAB (acetic acid bacteria), CH2 (WKGCH2), COL3 (WKGCOL3), FG (final grain), LA (lactic acid), AA (acetic acid), EtOH. (Ethanol) and YEAST.*

Factor	**Name**	**Level**	**Low Level**	**High Level**
X_1	**Agitation (rpm)**	54.37	23.43	136.57
X_2	**Temperature (°C)**	37.00	22.93	37.07
Solution Response	**Predicted Mean**	**Std Dev**	**95% CI low for Mean**	**95% CI high for Mean**
LAB-CH2	37868.2	3590.46	29588.61	46147.83
AAB-CH2	291.803	25.66	232.64	350.96
YEAST-CH2	22212.5	1982.37	17641.16	26783.86
LAB-COL3	43782.6	3230.75	36584.09	50981.20
AAB-COL3	141.483	34.92	60.95	222.01
YEAST-COL3	24560.7	5480.40	11922.85	37198.51
FG-CH2	93.6346	2.55	88.02	99.25
LA-CH2	7.87321	0.81	6.01	9.74
AA_CH2	0.383742	0.021	0.33	0.43
EtOH_CH2	14.9829	0.43	14.00	15.97
FG-COL3	100.699	1.47	97.31	104.09
LA-COL3	6.85966	0.58	5.53	8.19
AA_COL3	0.568533	0.077	0.39	0.75
EtOH_COL3	16.08	0.47	15.00	17.16

The variability in abundance was studied according to the modifications imposed, based on the changes observed under the same stirring and temperature conditions between the times of 48 and 96 hours (see Table F, Appendix 8.6), where it was noted that for all bacterial genera and in both grains there were no significant variations according to the calculation of standard deviations (SD). No significant variations in WKHCH2 in experiments 1 (23°C, 137 rpm), 4 (40°C, 80 rpm), and 5 (37°C, 23 rpm) and in experiments 6 (30°C, 0 rpm) and 8 (20°C, 80 rpm) also, in WKGCOL3, there was no significant variation for all the genera; in the case of WKGCH2, under the same condition 6, only the genera *Gluconobacter* and *Acetobacter* remained stable. In general, the *Acetobacter* and *Gluconobacter* did not show significant variations in all the experiments, indicating that these bacteria only increased their abundance at the beginning of the fermentation. Perhaps this last point can also be explained by the fact that the system did not initially reach a completely anaerobic environment, since the CO_2 produced during fermentation, especially by LAB and yeasts, displaces the oxygen initially present in the headspace of the flask. It can also be said that since these species are underrepresented, they cannot compete significantly for the available substrate; therefore, the abundance is not significant in any experiment, as it does not reach 1% at any point. The *Lactobacillus* generally showed greater variability under conditions 2 (30°C, 160 rpm) and 3 (37°C, 137 rpm) for both grains with a standard deviation above 2. This may indicate that operating

at temperatures above 30°C and vigorous agitation of the system may affect the growth of these microorganisms.

In the case of yeasts, the conditions where the greatest stability was observed in common for both grains were in experiment 3 (37°C, 137 rpm), 13 (30°C, 80 rpm), 5 (37°C, 23 rpm), 2 (30°C, 160 rpm), and 6 (30°C and 0 rpm), indicating that maintaining temperatures between 30 and 37°C may provide stability to yeasts regardless of agitation levels. *Saccharomyces* and *Dekkera* were the genera that showed significant variability in common for the two grains, and their growth was affected under conditions 1 (23°C, 137 rpm), 4 (40°C, 80 rpm), 7 (23°C, 23 rpm), and 8 (20°C, 80 rpm). It was observed that these microorganisms are strongly affected when operating under the most extreme temperature conditions, with a trend indicating that below 23 °C, this change seems to affect the growth of these two genera. According to the abundances found in the experiments for all genera in general (see Appendix 8.6), the abundances at 48 hours were greater or very similar to those at 96 hours; positive changes in abundance were observed only for *Zygotorulaspora* in WKGCH2 and for *Dekkera* in WKGCOL3. A similar case was observed for bacteria in general, where the average relative abundances between the two times were always less than 48 hours or very similar to those at 96 hours. It can be said that bacteria and yeasts have already reached a peak of growth after 48 hours and that even their representation may be affected, perhaps by the formation of products.

According to the abundance graphs (Figures 4.17 and 4.18), there is a noticeable trend of increasing abundance of *Lactobacillus* in WKGCH2 as the temperature increases to 40 °C, which is not observed for WKGCOL3. However, for WKGCOL3, after stirring, the bacteria show greater variations when operating at levels above 80 rpm, which also indicates that stirring leads to a destabilization of the system fermentation. According to the graphs showing the duplicated experiments at 48 and 96 hours, it can be observed that for WKGCOL3, the conditions that favored the increase in the abundance of *Lactobacillus* were 2 and 7, while for *Oenococcus* it was favorable in experiments 3 and 5. In the case of WKGCH2, increases were observed after 48 hours for *Lactobacillus* in the same conditions and also in condition 6. In the same grains, *Oenococcus* were favored only in condition 8.

For yeasts (Figures 4.19 and 4.20), stability of the abundance levels was observed when the temperature was maintained at 30 and 37 °C in both grains. A behavioral pattern can also be observed according to the agitation; when operating between 0 and 23 rpm and between 137 and 160 rpm, the yeasts seem to stabilize their abundances and do not vary significantly among the experiments carried out within this range of agitation, considering that this only applies to the experiments carried out at temperatures

between 30 and 37°C. The changes are more noticeable at 80 rpm and at extreme experimental temperatures. According to the graph showing the duplicated experiments at 48 and 96 hours, notable changes are observed for WKGCOL3 in conditions 1, 4, and 8, where the abundance was consistently negatively affected. In these experiments, the temperature conditions were either very low (below 23°C) or very high (40°C), and the system was always agitated above 80 rpm, suggesting that the different states evaluated in fermentations at temperatures far from optimal for the yeast, with significant agitation of the system, cause instability in the fermentation. For bacteria, it is also noted that there is no reproducibility between experiments conducted at the same agitation levels. The latter involves the identification of conditions that do not favor the stability of the consortium and that must be considered when making operational decisions. Given that in a process that aims to be standardized, it is more useful to keep the system under control in conditions that allow reproducibility, very noticeable changes common to both grains were generally observed when operating at temperatures far from the optimum for the yeasts (20°C and 40°C). This revealed trends of lower variability at temperatures between 30 and 37°C for both yeasts and bacteria. It is also noted that microbial groups are more affected in terms of stability when operating above 80 rpm.

In view of the results obtained from the analysis of the variability of the standard deviation and the results obtained from the global optimization analysis, it was determined that an operating point that allows the standardization and control of the process without causing changes in the system, and at which a fermented beverage with appropriate physical-chemical and microbiological characteristics can be obtained for the development of a potentially functional product with good market acceptance, is at a temperature of 37°C and a stirring speed of 54 rpm, maintaining an anaerobic system and using 80 g/L as the initial concentration of water kefir grains and 80 g/L of non-centrifugal sugar cane.

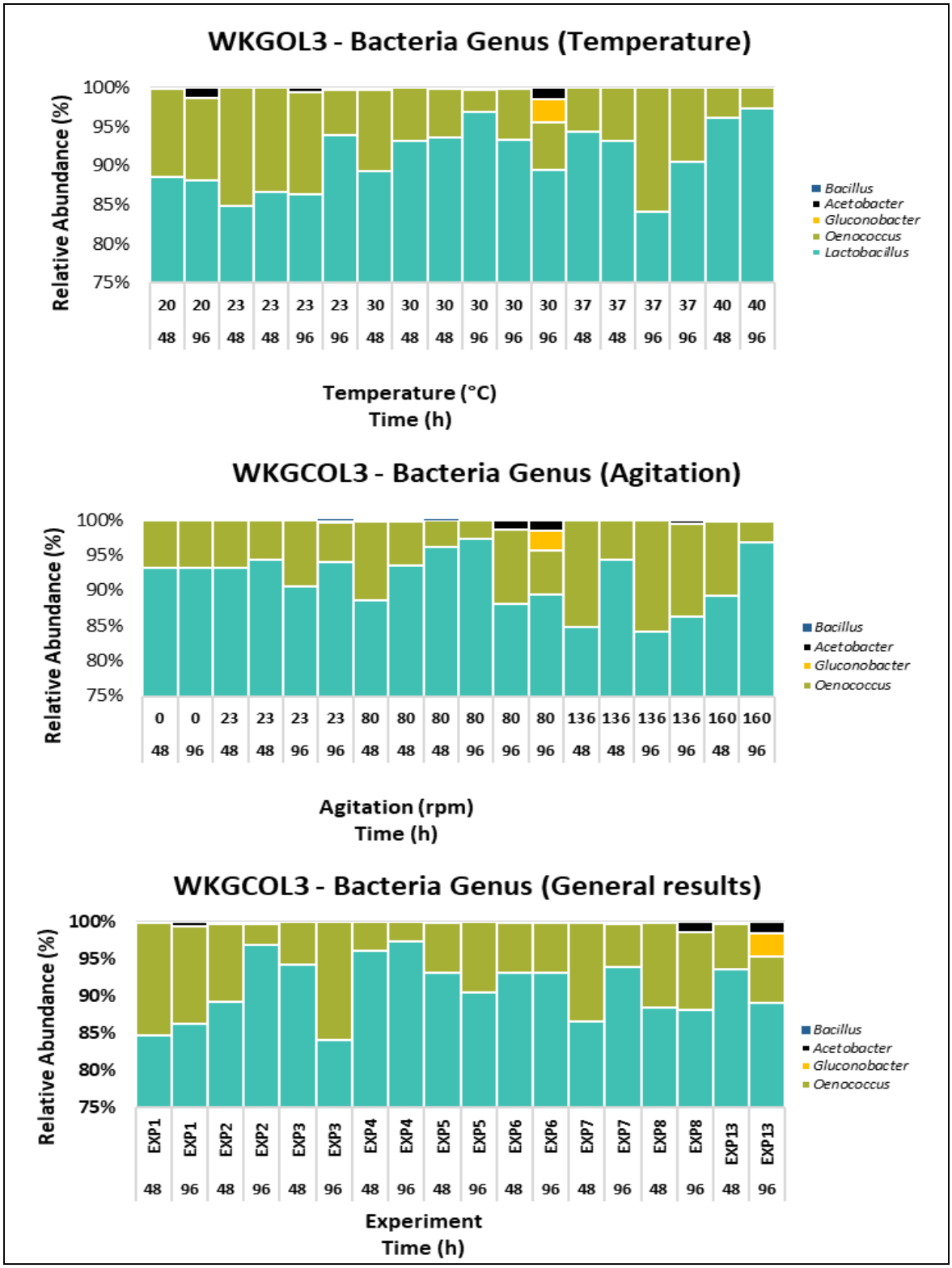

Figure 4.17. *Relative abundance (%) according to the metagenomic analysis performed for the dynamic study, indicated for each bacterial genus in WKGCOL3 grains. EXP (Experiment); (1,2,3… etc., is the experiment number).*

Figure 4.18. *Relative abundance (%) according to the metagenomic analysis performed for the dynamic study, indicated for each bacterial genus in WKGCH2 grains. EXP (Experiment); (1,2,3… etc., is the experiment number).*

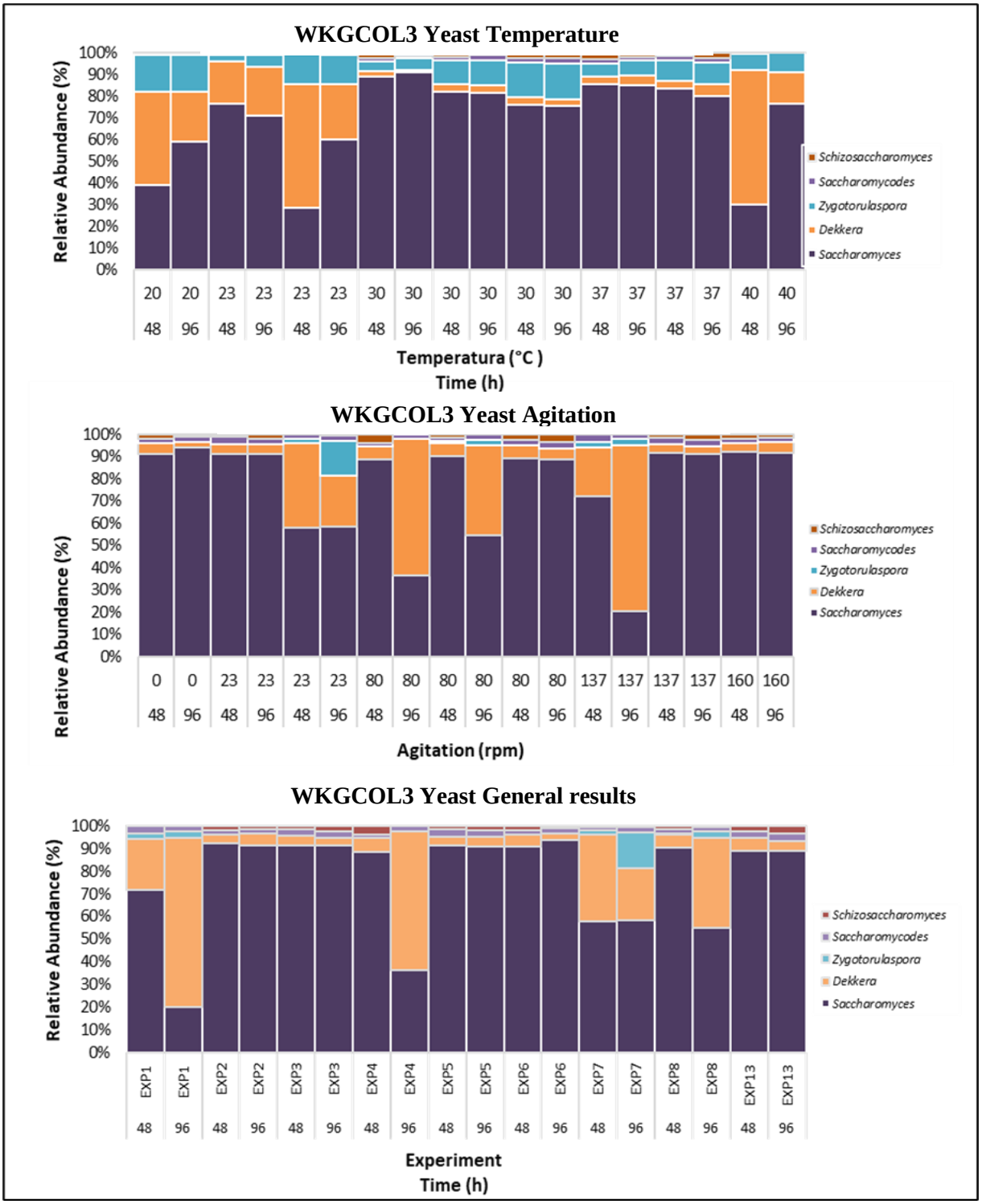

Figure 4.19. *Relative abundance (%) according to the metagenomic analysis performed for the dynamic study, indicated for each fungal genus in WKGCOL3 grains. EXP (Experiment); (1,2,3… etc., is the experiment number).*

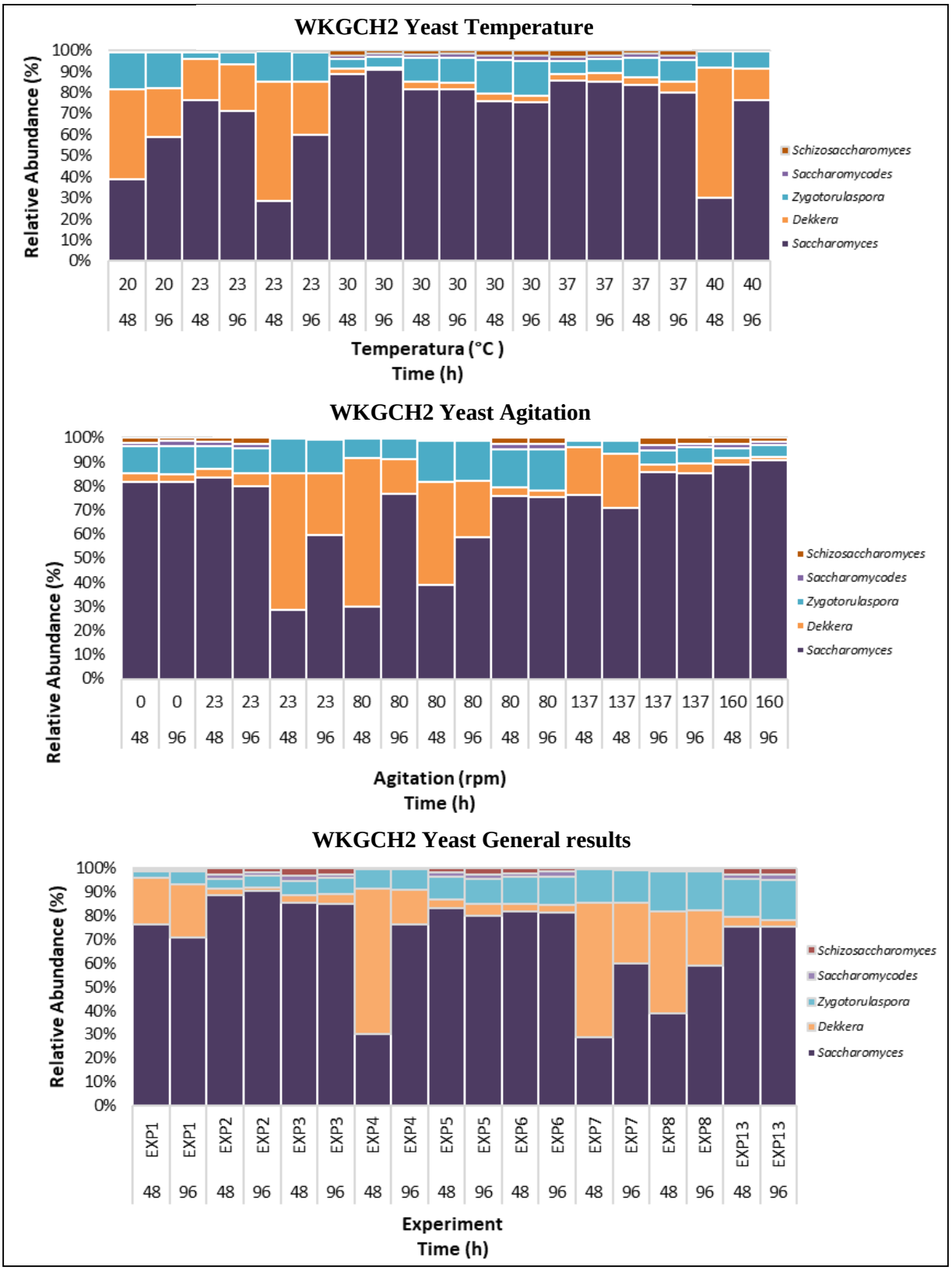

Figure 4.20. *Relative abundance (%) according to the metagenomic analysis performed for the dynamic study, indicated for each fungal genus in WKGCH2 grain. EXP (Experiment); (1,2,3… etc., is the experiment number).*

4.8. Changes in biomass growth and pH as a function of substrate change.

At the end of all fermentations, the final mass of the kefir grains was weighed, and the increase in biomass was calculated based on the amount of inoculum added at the beginning. The pH at the end of each fermentation step provided information on the growth of the grains and the subsequent production of ethanol, which promotes acidification of the medium. The pH in media containing fruit and/or sugar decreases due to the formation of lactic acid and acetic acid from ethanol. (Liu, Han, and Zhou 2011). The results of both experiments are presented in Tables 4.26, detailing the increase in WKG mass and pH at the end of each fermentation. For all the fermentations with Latin American grains carried out in Experiment 1, it was observed that the pH tended to decrease after each successive fermentation, reaching pH values of 3.32 for WKGCOL3 and 3.40 for WKGCH2 at step 8; in the case of WKGALE, the pH fluctuates without a defined trend (see Figure 4.21), which may indicate that the adaptation to the new substrate is slower than for the grains from Colombia and Chile.

In experiment 2, it is observed that the pH of WKGALE increases, reaching step 3 (with the new substrate, NCS) with a pH of 4.53, indicating that the pH is probably related to growth since the mass yield of the grains also showed a clear tendency to increase. This second experiment was carried out with the remaining grains used in experiment 1, which had already been previously adapted to the new substrate (NCS). For this reason, it may be that WKGALE began to stabilize and show a better adaptation to the substrate. As it continues to increase the mass yield of grains, it is known that at a higher pH the mass of water kefir increases (David Laureys *et al.,* 2019).

In the 1990s, Reiß (1990) proposed that there was at least one factor that promoted grain growth in figs, which could be calcium. More recent studies, such as that of Laureys *et al.,* (2019), have found that calcium content and buffering capacity affect grain growth; the higher the calcium content, the greater the buffering capacity and the greater the growth.

It is known that the calcium content of dried figs used in Europe is about 162 mg/100 g, which is much higher than that of other fruits used for this type of fermentation (Lynch *et al.,* 2012). (Lynch *et al.,* 2021; Fiorda *et al.,* 2017) In the case of unrefined sugars (NCS), this substrate can contain between 103 and 293 mg/100 g of calcium, along with other micronutrients such as potassium (531 mg/100 g) and phosphorus (58 mg/100 g) (Jaffé 2015; Guerra and Mujica 2010). Given this, the calcium and various micronutrients present in the NCS may have a positive effect that influences the growth of the kefir grains in this study, as these minerals can regulate the pH and consequently the growth of the grains (Zannini *et al.,* 2023; David Laureys *et al.,* 2019; Jaffé, 2015).

Table 4.26. *Summary table of the results of the first and second substrate change experiment. The data corresponds to the increase in biomass (%) of the kefir grains and the final pH at each stage of fermentation.*

First experiment			
Biomass increase (%)			
Step	***WKGCOL3***	***WKGCH2***	***WKGALE***
Step 01	57.16	51.67	5.26
Step 02	19.06	40.43	4.59
Step 03	22.73	23.95	17.00
Step 04	28.55	29.19	33.04
Step 05	17.31	20.25	28.37
Step 06	17.61	20.84	35.98
Step 07	7.81	16.19	40.34
Step 08	15.17	26.24	53.08
pH			
Step	***WKGCOL3***	***WKGCH2***	***WKGALE***
Step 01	4.18	4.00	3.78
Step 02	4.07	3.93	3.53
Step 03	3.76	3.44	3.91
Step 04	3.46	3.60	3.86
Step 05	3.58	3.60	3.90
Step 06	3.52	3.59	3.97
Step 07	3.61	3.54	4.14
Step 08	3.32	3.40	3.84
Second experiment			
Biomass increase (%)			
Step	***WKGCOL3***	***WKGCH2***	***WKGALE***
Step 01	53.98	38.03	26.38
Step 02	50.30	34.46	55.43
Step 03	52.80	39.10	70.67
pH			
Step	***WKGCOL3***	***WKGCH2***	***WKGALE***
Step 01	4.00	3.84	3.51
Step 02	3.52	3.59	4.36
Step 03	3.53	3.67	4.53

According to the observations in experiment 1, the greatest increases in biomass were obtained with NCS as substrate. For Latin American grains, a greater increase in biomass was observed with the conventional substrate during fermentation steps 1 and 2, with increases of 57% and 52% for WKGCOL3 and WKGCH2, respectively. This could indicate that the adaptation to a different substrate had a direct effect on the bio augmentation of these grains during the subsequent steps (6–8), where it was not evident that the new substrate used (sucrose with lemon and dried figs) promoted an increase in the mass of the grains. Biomass yields ranged from 7% to 28% for WKGCOL3 and from 16% to 29% for WKGCH2

(see Figure 4.21) between steps 3 and 8. In contrast, WKGALE increased its grain increment from a minimum of 4.59% (in conventional substrate) to 53% in NCS, with the latter being the maximum percentage reached in step 8. It is noteworthy that WKGALE gradually increased after each fermentation step in the new substrate, from 17% in step 3 to 53% in the final step. This indicates that the microbial community begins to adapt to the new substrate during successive fermentations and that there are likely other factors inherent in the composition of the NCS that promote growth.

In Experiment 2, the grain yield was equally favorable with the use of NCS; in the case of WKGALE, the grain mass showed higher yields with NCS. The maximum value observed (see Table 4.26) was 71%, which corresponds to the final stage of fermentation. (Step 3). For WKGCOL3 and WKGCH2, it was observed that the biomass yields stabilized (see Figure 4.22), with percentage differences of no more than 2% but higher than the yields obtained in Experiment 1.

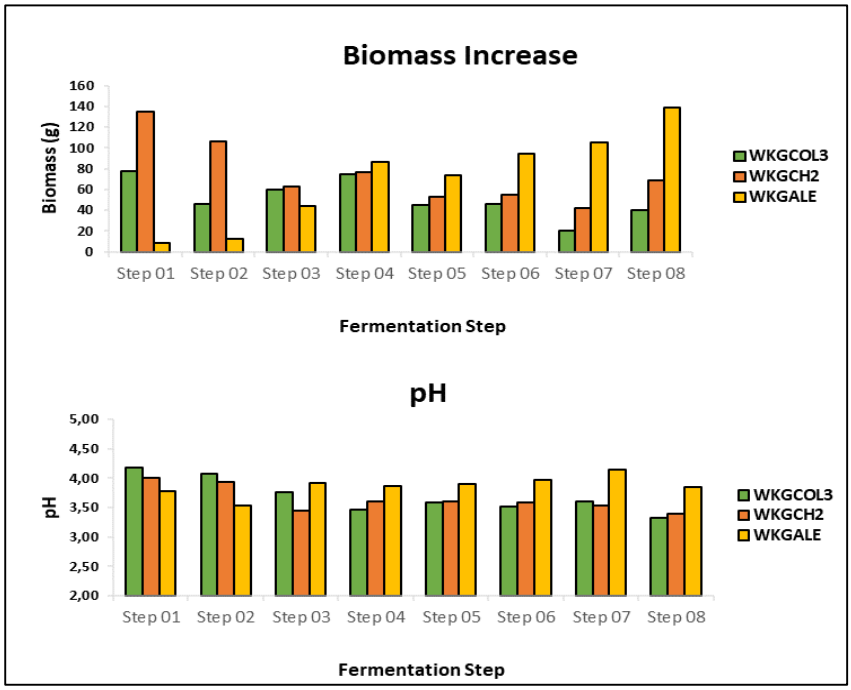

Figure 4.21. *Biomass Increase and pH change during 8 steps fermentations – first experiment. Steps 1 and 2 refer to the fermentations with conventional substrate and the next 6 fermentation steps (steps 2 to 8) refer to the fermentations with the new substrate.*

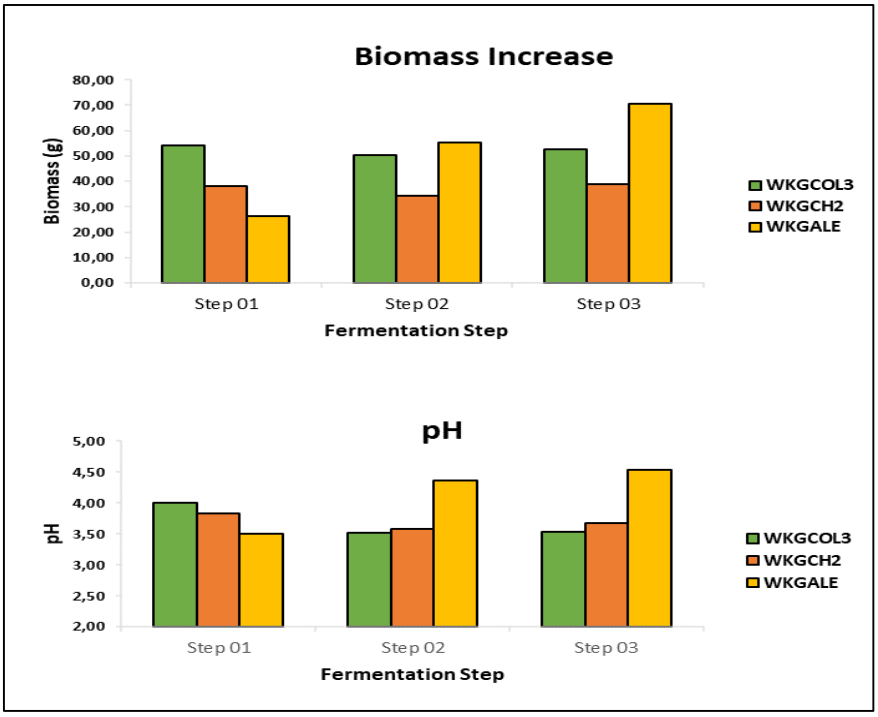

Figure 4.22. *Biomass Increase and pH change during 3 steps fermentations – second experiment. Fermentations in step 1 were performed with the conventional substrate and steps 2 and 3 were performed with the new substrate.*

It can be concluded that NCS is a more suitable substrate for fermentation of kefir grains; this may be because this unrefined sugar contains more micronutrients than those provided by the conventional European substrate (refined sugar with dried figs). Making a comparison with the operating conditions used in the experiment for the aforementioned optimization (CCD), it can be concluded (see Appendix 8.7, table A) that with NCS, at temperatures between 23 and 30°C, grain yields between 52 and 88% can be obtained, much higher than those reported by other authors using European substrates (Laureys *et al.,* 2017) and what was obtained in this thesis (see table 4.26).

4.8.1. Microbial group enumeration and species identification using culture-dependent techniques.

The numbers of viable microorganisms cultivated in MRS, YPG, and mTY growth media remained relatively stable throughout all fermentation steps in both Experiment 1 and Experiment 2, with average numbers for MRS cultures in Experiment 1 of 6.03*10^7, 1.05*10^7, and 2. 7*10^7 CFU/mL and 5.8*10^7, 7.5*10^7 and 4.6*10^7 CFU/mL in Experiment 2 for WKGCOL3, WKGCH2 and WKGALE, respectively (see Table 4.27), indicating that the substrate change did not significantly affect the

abundance of microbial groups such as Lactobacillus and Acetobacter. The viable counts of molds and yeasts in the YPG medium in Experiment 1 also remained stable at levels of 1.95*10^7, 1.84*10^7, and 1.57*10^7 CFU/mL for the WKGCOL3, WGCH2, and WKGALE grains, respectively. In experiment 2, they also showed stable values of 3.1*10^7, 8.9*10^7, and 2.6*10^7 CFU/mL for the same grains. It is assumed that Bifidobacterium grows in mTY medium, but it only showed growth in steps 1, 5, 6, and 7, with values also very close to the order of 10^6 for all grains; other studies have reported counts of *Bifidobacteria* also in the order of 10^6. (A. Gulitz *et al.,* 2013). Studies of water kefir fermentations have reported growth values of LAB in kefir liquors of 10^6 CFU/ml (Patel *et al.,* 2022) and in the order of 10^7 and 10^8 CFU/ml, and of yeasts up to 10^7 CFU/mL (Moretti *et al.,* 2022) which agrees with the findings of the present study. In general, it has been reported that the population of LAB exceeds that of yeasts in WKG (Lynch *et al.,* 2021), however, some studies have reported similar populations or have shown the opposite, i.e., predominance of the yeast population over LAB (Moretti *et al.,* 2022). Studies of water kefir fermentations have reported growth values of LAB in kefir liquors of 10^6 CFU/ml (Patel *et al.,* 2022) and in the order of 10^7 and 10^8 CFU/ml, and of yeasts up to 10^7 CFU/mL (Moretti *et al.,* 2022), which is consistent with the results of the present study. In general, it has been reported that the population of LAB exceeds that of yeasts in WKG (Lynch *et al.,* 2021), but some studies have reported similar populations or have shown the opposite, i.e., the predominance of the yeast population over LAB (Moretti *et al.,* 2022).

In the MALDI-TOF analysis performed, 428 colonies were analyzed, of which 232 microorganisms were identified and 186 could not be classified according to the database included in the instrument. The data were processed as described in Kern *et al.,* 2013 and Usbeck *et al.,* 2013. Sample identification was performed using the MALDI BioTyper 3.0 software (Bruker Daltonics, Bremen, Germany), which includes a reference database of 4,111 microbial spectra, of which 242 belong to the genera *Lactobacillus, Pediococcus, and Leuconostoc*, covering approximately 100 different species. A logarithmic score >2.0 indicated successful identification of a sample at the species level according to the manufacturer's instructions. Clin ProTools 2.2 (Bruker Daltonics, Bremen, Germany). Figures 4.23 and 4.24 show that microbial species such as *L. paracasei, L. harbinensis, Oenococcus kitaharae, L. nagelli, L. satsumensis, Zymomonas mobilis* and *L. parabuchneri* were present in stage 1 MRS cultures (fermentation on conventional substrate). In the three grains, *L. paracasei* was present in the fermentation with the conventional substrate in percentages that did not exceed 5.9%; *Oenococcus kitaharae* was found in the Latin American grains with a percentage abundance of 50% for WKGCOL3 and 41% for WKGCH2, but this species disappeared when the substrate was changed to the one used in Germany. *Oenococcus* species have recently been identified as a novelty in microbiota associated with

water kefir, as new species of this genus have been identified in recent studies. (Verce, De Vuyst y Weckx 2019; 2020). It is important to note that NCS may play a role in increasing this microbial group.

Table 4.27. *Summary table of microbial enumeration results. Data are for colony forming units (CFU/mL) First experiment, steps 1 and 2 - conventional substrates, and the following 6 fermentation steps (steps 2 through 8) refer to the substrate change. In the second experiment, step 1- conventional substrate, while steps 2 and 3 were carried out with the new substrate. In the second experiment, Bifidobacterium showed no growth.*

First experiment			
Viable counts on growth MRS media (CFU/mL)			
Step	***WKGCOL3***	***WKGCH2***	***WKGALE***
Step 01	2.52E+07	1.62E+07	3.76E+07
Step 02	1.00E+05	5.40E+06	3.30E+06
Step 03	2.65E+07	5.03E+07	4.70E+06
Step 04	1.46E+07	4.85E+06	3.05E+06
Step 05	2.91E+07	3.34E+07	1.36E+07
Step 06	3.16E+07	5.75E+07	2.66E+07
Step 07	3.92E+07	3.96E+07	2.03E+07
Step 08	6.03E+07	1.05E+07	2.07E+07
Viable counts on growth YPG media (CFU/mL)			
Step	***WKGCOL3***	***WKGCH2***	***WKGALE***
Step 01	8.50E+06	2.16E+07	3.86E+07
Step 02	6.90E+06	1.28E+07	4.10E+06
Step 03	3.01E+07	2.33E+07	3.10E+06
Step 04	3.12E+07	1.43E+07	1.77E+07
Step 05	1.38E+07	2.51E+07	2.70E+06
Step 06	1.61E+07	1.04E+07	1.71E+07
Step 07	1.36E+07	1.05E+07	1.70E+06
Step 08	3.54E+07	2.89E+07	4.02E+07
Viable counts on growth mTY media (CFU/mL)			
Step	***WKGCOL3***	***WKGCH2***	***WKGALE***
Step 01	5.10E+07	6.36E+07	9.45E+06
Step 02	-	-	-
Step 03	-	-	-
Step 04	-	-	-
Step 05	9.00E+05	1.50E+06	3.40E+06
Step 06	1.30E+06	4.00E+05	3.10E+06
Step 07	7.00E+05	5.00E+05	3.30E+06
Step 08	-	-	-
Second experiment			
Viable counts on growth MRS media (CFU/mL)			
Step	***WKGCOL3***	***WKGCH2***	***WKGALE***
Step 01	9.30E+07	6.20E+07	6.70E+07
Step 02	1.00E+05	5.40E+06	3.30E+06
Step 03	8.10E+07	1.57E+08	6.70E+07
Viable counts on growth YPG media (CFU/mL)			
Step	***WKGCOL3***	***WKGCH2***	***WKGALE***
Step 01	1.40E+07	1.28E+08	3.30E+07
Step 02	6.00E+07	1.26E+08	2.70E+07
Step 03	1.0E+07	1.30E+07	1.70E+07

Zymomonas mobilis appeared in the first step of fermentation of WKGALE with conventional substrate, which could indicate that this microorganism was potentially present in the added fruit. Significant amounts of unidentified microorganisms were found in the grains from Colombia and Chile (30% and 47%, respectively), which represents an opportunity to discover new species of technological and/or

probiotic interest. When the substrate was changed (steps 2 and 3), it was observed that the diversity in the grains decreased as fewer species were identified, with *L. nagelli, Zymomonas mobilis* and *Oenococcus kitaharae* being the most prominent; it was also noted that the number of unidentified species increased significantly, especially in WKGCH2, and *L. nagelli* appeared when the conventional European substrate was used. The presence of *Zymomonas mobilis* persisted in all the grains, suggesting that its presence is due to some form of contamination. *Oenococcus kitaharae* tended to disappear in grains WKGCOL3 and WKGCH2 as the substrate change progressed to step 3, dropping from 41% to 5% abundance in WKGCH2. These results indicate that the microbiota of the grains changes significantly depending on the substrate to which the consortium adapts (Figures 4.23 and 4.24).

The main AAB species identified were *Acetobacter loavaniensis, Acetobacter ghanensis, Acetobacter fabarum, and Acetobacter orientalis. A. lovaniensis* was found only in WKGCOL3 and WKGCH2 and decreased significantly from 73% to 11% in WKGCH2 due to the substrate change. *A. ghanensis* was identified only once in WKGCH2 during the first fermentation step and accounted for 10.5%; *A. fabarum* and *A. orientalis* were also identified particularly in the fermentations of WKGCOL3 and WKGCH2 in conventional substrate with 60% and 32%, respectively. The identification of *acetobacteria* in this study is important because in the metabarcoding analysis study, no *acetobacteria* could be identified at the species level. This may be due to the fact that the anaerobic operating conditions during the optimization did not allow a good development of *acetobacteria*, contrary to what happened in this experiment (substrate exchange for biomass increase analysis), where the fermentations were carried out at room temperature and in aerobiosis.

These *Acetobacter* species have also been reported by other authors as characteristic microbiota of this type of grain (David Laureys *et al.,* 2017; Anna Gulitz *et al.,* 2011b; Magalhães *et al.,* 2010). *Gluconobacter oxydans* was found in WKGCH2 in the second step of fermentation and is also a genus reported by other authors. (David Laureys *et al.,* 2017). Although it was expected that yeasts and molds would predominate in the YPG medium, surprisingly there was greater growth of lactic and acetic acid bacteria; the only yeast species identified was *Saccharomyces cerevisiae* in WKGALE during the first and last stages of fermentation, with an abundance of 25% and 28%, respectively, a yeast that is widely known to be ubiquitous in WKGs. (Lynch *et al.,* 2021; Patel *et al.,* 2022).

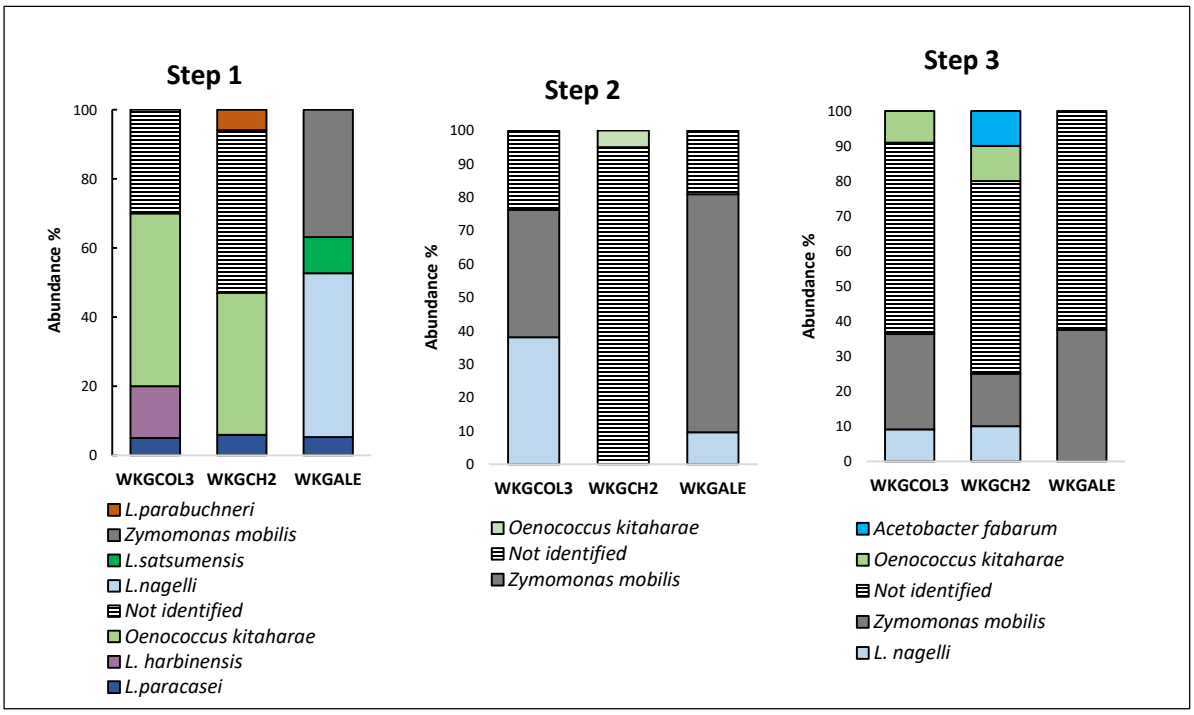

Figure 4.23. *Abundance of microbial species (%) in culture media MRS—second experiment. The fermentations in step 1 were performed with the conventional substrate, while steps 2 and 3 were performed with the new substrate.*

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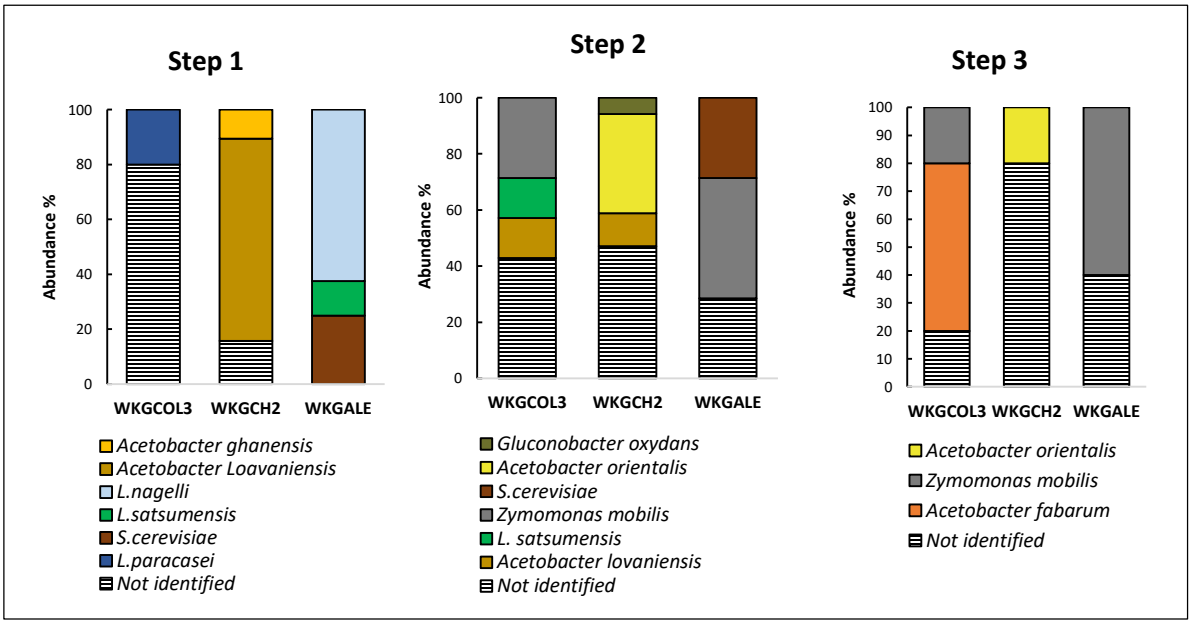

Figure 4.24. *Abundance of microbial species (%) in the YPG culture medium—second experiment. The fermentations in step 1 were performed with the conventional substrate, while steps 2 and 3 were performed with the new substrate.*

4.8.2. Microorganisms identified by 16S rRNA gene sequencing

With the aim of isolating microorganisms with potential probiotic and/or technological properties from these cereals, 6 strains were selected from a total of 32 lactic acid bacteria collected from cultures grown on MRS and mTY media. The aim was to isolate *Lactobacillus, Bifidobacterium* and *Oenococcus*. In the case of *Oenococcus,* there were precedents for its possible presence as had been identified in a previous analysis by MALDI-TOF. The following microorganisms were identified (see Table 4.28), with very close results according to the database used for their identification: *Bifidobacterium aquikefiri, Liquorilactobacillus nagelii and Lactobacillus hilgardii*. The microbial species and amplified fragments were verified by sequencing by Eurofins Genomics (Ebersberg, Germany) and comparative searches using the Basic Local Alignment Search Tool (BLAST) in the GenBank/EMBL/DDBJ database. The identity of these organisms was determined by aligning the partial sequence of the 16S rRNA ribosomal gene, analyzing the maximum score, the query coverage and the percentage of identity explaining the maximum coverage of all sequences, as well as the e-value, which defines that there is a very low probability that the alignments occurred by chance. The bacterium *Liquorilactobacillus nagelii* has recently been reported as a microorganism present in water kefir grains in NCS solutions (Bozkir *et al.,* 2014; Rodriguez *et al.,* 2014); this organism may be present on the substrate and perhaps not indigenous to the microbiota inherent to the grain as such.

Table 4.28. *This table describes the microorganisms found by culture-dependent techniques and 16S rRNA analysis of isolated strains. The maximum score, the query coverage, the e-value and the percentage of identity found in the NCBI database can be observed.*

Organism description	***Scientific name***	***Max. Score***	***Query Cover***	***E-value***	***Percent identity***
Lactobacillus hilgardii strain AF15 16S ribosomal RNA gene, partial sequence	*Lentilactobacillus hilgardii*	1851	100%	0	99,70%
Liquorilactobacillus nagelii strain TMW 1.1827 chromosome, complete genome	*Liquorilactobacillus nagelii*	1718	100%	0	99,89%
Bifidobacterium aquikefiri strain LMG 28769 16S ribosomal RNA, partial sequence	*Bifidobacterium aquikefiri*	2010	100%	0	99,64%

4.9. Project proposal for the development of NCS fermented beverage with water kefir grains, based on the results obtained in this thesis.

The project was formulated according to the guidelines and requirements requested by the National Agency for Research and Development (ANID) of the Government of Chile for the VIU (Valorization of Research in the University) 2019 call. The project aims to test some functional properties of fermented water kefir drinks with probiotic potential, for which bottled kombucha beverage manufacturers, dairy probiotics such as yogurts, and prescription probiotics were considered the main competitors in the market. The proposed solution aims to offer a standardized and optimized product in its process and production parameters, ensuring the preservation of the final product in terms of taste, microbial viability, and its functional properties.

The benefit of differentiating for the user of this standardized product will be the possibility to consume a consistently high-quality beverage at a low cost, with a pleasant taste and functional properties that can be attributed to the microorganisms it contains. Production could be carried out in a certified food facility with reactors and packaging lines suitable for the expected sales volume, employing qualified personnel to operate the reactors and machinery. If there is no capital available to purchase equipment and rent space, the production service (contract manufacturing) could be outsourced to a company that produces beer, juices or water, which would provide the service according to the process conditions and maintain industrial confidentiality.

4.9.1. Main characteristics of the product.

- Ensuring that the beverage is kept in good conservation and quality conditions by monitoring and maintaining physicochemical and microbiological sensory parameters, as well as temperature ranges and times during processing and storage.
- The use of sustainable and/or reusable packaging that guarantees the microbiological and physicochemical quality of the product and enhances the shelf life and viability of microorganisms.
- Possess certifications to comply with legislation on the distribution of water and food.
- The know-how is owned by the company and allows the reproducibility of this product in other countries where unrefined sugar is available. It is quite possible that the use of unrefined sugar will allow the product to be manufactured in almost all Latin America, since countries such as Brazil, Bolivia, Peru, Venezuela, Ecuador, Colombia, Chile and Mexico produce this type of substrate.

-It is a proven method of standardization and production of water kefir that allows the production of a standard beverage with a stable starter culture, allowing the sustainability of the process over time and, consequently, the maintenance of product supply.

4.9.2. Critical milestones to reach the proposed stage of development

Tests at different process volumes, fermentation stability tests, taste tests, sensory analysis of consumer acceptability, viable microorganism content in the beverage, physicochemical tests, immunobiotic tests.

4.9.3. Barriers to entry for the product

The main barriers to entry are having the necessary health resolutions for the sale and production of the product, having quality validations from consumers, brand generation, and market penetration, especially in foreign markets that have recognized brands and a history in terms of packaged products. Finally, it is necessary to carry out a commercial management strategy to attract customers and achieve significant market share.

4.9.4. Market segment, customer group or product user

The current global probiotics market is worth $32.06 billion, of which 71% is yogurt and 16% is dairy beverages. This market is currently expanding and has one of the highest growth rates in the global functional food market. In Latin America, this market reaches USD 5.58 billion with a growth rate of 6.74%. The addressable market is $243.5 million in Chile, $2.9 billion in Brazil and $1.56 billion in Mexico. The global probiotics market size exceeded USD 2 billion in 2018 and is estimated to grow at a CAGR of over 7.8% between 2023 and 2028 (see Figure 4.25). Increasing consumer inclination towards probiotic foods and beverages and growing awareness about the potential benefits of probiotic strains can boost the global demand for industry.

Figure 4.25. *Global Probiotics Market Size, by Product, with a CAGR (Compound Annual Growth Rate), 2023 - 2028 Source: Technavio.*

The global probiotics market is expected to be valued at $32 billion by 2023, according to Technavio. Chilean consumers are also increasingly turning to higher-quality formulations and are interested in getting benefits from the products they consume. This, along with the implementation of the Food Labeling Law in June 2016 (Law No. 20,606 on the Nutritional Composition of Foods and Their Advertising), has, in one way or another, led our society to take a new approach to the foods they consume. Despite the economic slowdown in recent years, data from Euromonitor International shows that the health and wellness market in Chile has grown by 11.3% between 2010 and 2015, with the categories of food intolerances and fortified/functional foods experiencing the highest growth in the same period (15.3% and 15.4%, respectively), followed by the category of "reduced foods" with 9.6% and "naturally healthy foods" with 7.8% (% CAGR). The position and opportunities for Chilean products that have been identified (according to the ProChile 2018 report) and that have the greatest opportunities are the manufacturers of products that offer: Energy/Sports Nutrition; Energy Drinks, High Protein Bars; Immune Support—Supplements. Digestive health: prebiotics and probiotics; healthy foods or snacks. As shown in Figure 4.26, there are trends in the types of foods that Chileans are currently looking for, with probiotics being part of the functional foods category, accounting for 42%. This indicates that there is a significant opportunity to enter the market with many possibilities.

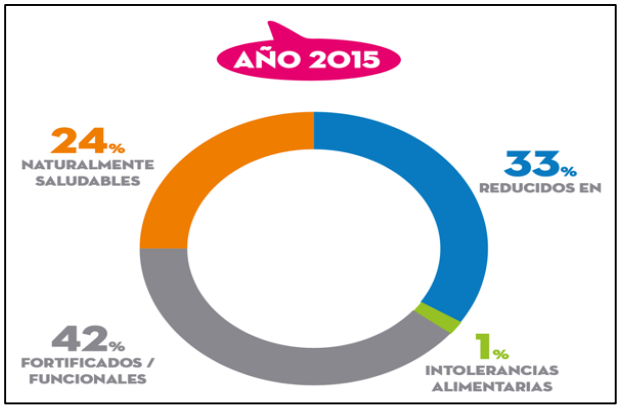

***Figure 4.26**. Percentage of health and wellness sales by category as of 2015 in Chile. Source: Technavio*.

Currently, there are different segments of the population that suffer from conditions such as a weakened immune system (20% of people in Chile suffer from irritable bowel syndrome, according to the Las Condes Clinic), intestinal problems (14.5% of deaths in adults between 65 and 79 years of age are due to digestive diseases, and for those over 80 years of age it is 10.6%), allergies and food intolerances that require the recovery of the intestinal flora, and one of the ways to achieve this is through the consumption of probiotics. It is then the older adult population between 65 and 80 years old, a special segment to be the most important potential customers of this product. According to reports in Chile, it is estimated that 50% of the adult population is lactose intolerant; they also report that 20% suffer from irritable bowel disease, which is more common in women between 20 and 40 years old. Adult women are therefore defined as a second consumer segment.

Schoolchildren between the ages of 5 and 10) are another segment where this product can meet a need in terms of the immune deficiency that many children develop due to poor eating habits. The consumption of probiotics can reduce school absenteeism since the consumption of probiotics strengthens the immune system, for example, the Tupahue School, in the population of La Bandera (municipality of San Ramón), serves 318 children. These are children with a vulnerability index of 81.7% who often have a low school attendance rate, mainly due to chronic diseases. In conclusion, the segments were defined as being served by the emporiums, cafeterias and restaurants that offer organic, natural, functional, or vegan products, company canteens where adults commonly work (with a high probability that around 50% may suffer from some type of gastrointestinal disease) and student canteens in the country (children with immune deficiency). In Chile there are more than 16,000 restaurants and cafeterias, more than 5,000 business casinos and 9,000 student casinos. It is known that emerging venues with functional product offerings and/or special features are still growing and their percentage representation in the current market is not very clear, as many are not well publicized. It is therefore a

challenge to approach these types of restaurants and cafés to introduce our products. As a projection, we also plan to penetrate supermarkets and mini markets, as all the segments converge in these places.

4.9.5. Critical Resources and Key Activities

The business model (see Figure 4.27) envisages the creation of a research-based company dedicated to the sale of packaged beverages, with an associated after-sales service, as well as the company's commitment to research and development of new products, processes, and methodologies that will allow it to continually expand its offer and value proposition to the market. The customers identified in the first phase are: shops, cafes, and restaurants, as well as university canteens, corporate cafeterias, and other similar establishments that serve many people daily. The direct users are the staff working in the various cafes and dining halls, such as baristas, waiters, kitchen staff, etc., while the indirect users are the customers of these places who choose to consume this beverage, which will come from purchases made in stores or supermarkets. The critical actors were identified as suppliers of unrefined sugar, fruit, and flavoring components; food casino dealers; Junaeb; Junji; restaurant and/or cafe chains that serve for sale; and transportation companies for distribution. In addition, the following key partnerships were defined to be established: companies dedicated to the sale and marketing of items for cafes, restaurants, and similar establishments; Junaeb, Sodexo, and/or Aramark; and school and/or university chains. The key activities for the development of the project include R&D, marketing, commercialization, production (working capital), quality control, and technical service for the equipment.

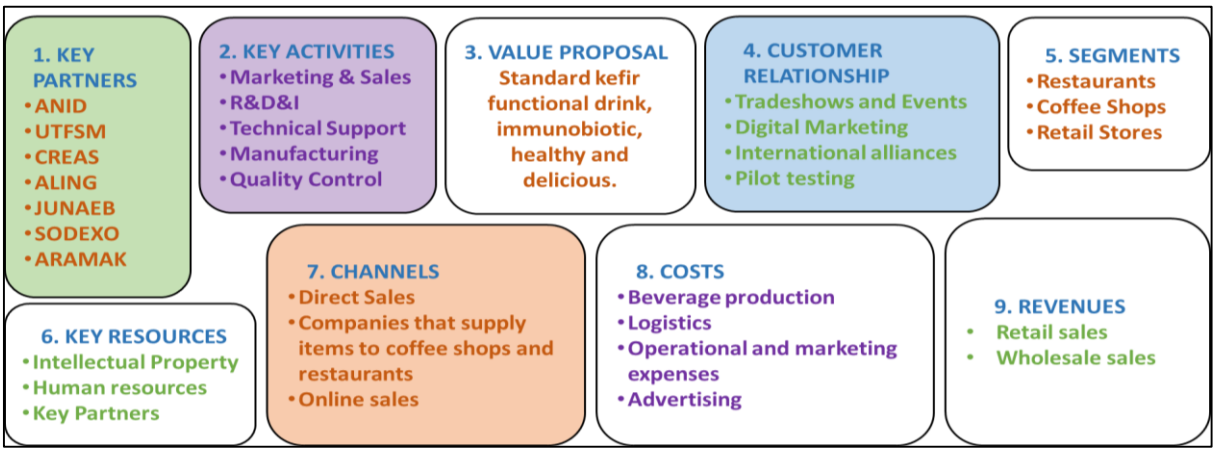

Figure 4.27. *Shows the description of the business model, identifying the indirect benefits related to the associated markets of the goods or services developed from the technology, which can be transformed into socio-economic benefits.*

4.9.6. Assessment of the value and economic viability of the project

According to a segmentation by age and nutritional needs (strengthening of the immune system and help with inflammatory bowel diseases), in the first year 10 establishments mainly located between the V region and the metropolitan region will be targeted (shops, restaurants and cafes offering functional and/or vegan food), 4 student restaurants serving 1,000 students per day (taking into account 8 levels, 2 courses per level, 45 students per course) and 4 company restaurants serving 500 people per day (see Table 4.29). By the 4th year, 300 business establishments, cafes and commercial restaurants, 20 student restaurants (colleges and universities), and 20 company restaurants will be reached. Direct channels will also be sought to market the product in supermarkets, mini-markets and neighborhood shops between the V region and the metropolitan region, with commissions on sales to the latter. The goal is to link up with 10 neighborhood stores in the first year, gradually doubling the number by year 4. As for supermarkets and mini-markets, it is hoped to connect with at least 2 of them in year 1, also gradually doubling the target by year 4, assuming that in the first year each company restaurant will reach 20% of workers who want healthy food (100 units per month), the same basis will be used to reach 20% of children in each student restaurant (200 units per month), and finally 100 units per month for each store, coffee shop, and/or restaurant, which is the target amount to encourage sales commissions. For neighborhood stores, the sales goal is 50 units per month to increase sales by 30% per year. For supermarkets and mini markets, the goal is to sell 100 units per month in the first year, again with a progressive increase of 30% for new supermarkets and convenience stores.

Table 4.29. *Number of beverage bottles in sales per year*

Sales (Number of bottles)	**Year 0**	**Year 1**	**Year 2**	**Yeas 3**	**Year 4**
Emporiums, Coffee shops and Restaurants	12,000	99,630	186,030	273,630	360,030
Student restaurants (1.000 people)	9,600	19,200	28,800	38,400	48,000
Company restaurants (500 people)	4,800	9,600	14,400	19,200	24,000
Neighborhood shops	6,000	6,015	12,015	24,015	48,015
Supermarkets and mini markets	2,400	2,430	4,830	9,630	19,230
Total	**34,800**	**136,875**	**246,075**	**364,875**	**499,275**

Using current market prices for various inputs, equipment, and leases of production space, the estimated cost of producing 400-ml beverages would be as follows:

Table 4.30. *Production costs per year [CLP].*

Value of each requirement (CLP)	Year 0	Year 1	Year 2	Year 3	Year 4
100 L Bioreactors	2,989,440.00	300,000.00	300,000.00	300,000.00	300,000.00
Bottling line	3,321,600.00	250,000.00	250,000.00	250,000.00	250,000.00
Operational human resources	11,538,000.00	11,538,000.00	11,538,000.00	11,538,000.00	11,538,000.00
Working capital	50,368,800.00		124,143,200.00	-	-
Administrative costs	3,000,000.00	3,000,000.00	3,000,000.00	3,000,000.00	3,000,000.00
Researcher R&D&I	1,500,000.00	1,500,001.00	1,500,002.00	1,500,003.00	1,500,004.00
Unrefined sugar	4,017,103.20	15,800,028.75	28,405,421.55	42,118,980.75	57,633,310.35
Cidrón	1,672,575.00	6,578,554.69	11,826,979.69	17,536,804.69	23,996,404.69
Maqui	260,826.00	1,025,878.13	1,844,332.13	2,734,738.13	3,742,066.13
Water	25,213,574.40	99,169,770.00	178,288,227.60	264,362,154.00	361,738,717.20
Packaging	904,800.00	3,558,750.00	6,397,950.00	9,486,750.00	12,981,150.00
Sanitary Resolution	90,400.00	-	-	90,400.00	-
Local Lease	1,500,000.00	1,500,000.00	1,500,000.00	1,500,000.00	1,500,000.00
Total	**106,377,118.60**	**144,220,982.56**	**368,994,112.96**	**354,417,830.56**	**478,179,652.36**

Table 4.30 shows the derived expenses that come from the cost of production of beverages, operators, and administrative staff (human resources). As shown in Table 4.32 below, there is a need for additional investment as capital investment for the first year of operation to then generate a new investment in the third year to support the process of consolidation of sales in Chile and commercial expansion to foreign markets. Operating costs, mobilization costs, and per diems (marketing costs).

Table 4.31. *Sales, marketing, and operating expenses per year [CLP].*

	Year 0	Year 1	Year 2	Year 3	Year 4
Other variable expenditure (CLP)					
Marketing	8,000,000	28,000,000	60,000,000	100,000,000	135,000,000
Domestic marketing costs	3,600,000	3,600,000	4,800,000	4,800,000	6,000,000
Other fixed costs (CLP)					
Operating Expenses	268,800	960,000	2,112,000	3,456,000	4,608,000
Other Costs (CLP)					
International marketing costs	10,000,000	10,000,000	20,000,000	20,000,000	35,000,000
Total	21,868,800	42,560,000	86,912,000	128,256,000	180,608,000

Table 4.32. *Revenue from sales of beverage bottles / year [CLP].*

	Sales revenue / year (CLP)				
Sales	**Year 0**	**Year 1**	**Year 2**	**Year 3**	**Year 4**
Emporiums, Coffeeshop and Restaurants	18,000,000	149,445,000	279,045,000	410,445,000	540,045,000
Student restaurants (1.000 people)	14,400,000	28,800,000	43,200,000	57,600,000	72,000,000
Company restaurants (500 people)	7,200,000	14,400,000	21,600,000	28,800,000	36,000,000
Neighborhood shops	9,000,000	9,022,500	18,022,500	36,022,500	72,022,500
Supermarkets and mini markets	3,600,000	3,645,000	7,245,000	14,445,000	28,845,000
Total	52,200,000	205,312,500	369,112,500	547,312,500	748,912,500

Below is a summary of the contributions obtained over the 4-year evaluation for the venture and the operating conditions presented.

Table 4.33. *Summary of financial flows obtained over 4 years [CLP]*

FLOWS [CLP]	Year 0	Year 1	Year 2	Year 3	Year 4
Revenues	52,200,000	205,312,500	369,112,500	547,312,500	748,912,500
Costs	87,475,200	170,240,000	347,648,000	513,024,000	722,432,000
			Net results		
	-35,275,200	35,072,500	21,464,500	34,288,500	26,480,500

Analyzing the cash flow presented over a period of 4 years and with a discount rate of 15%, considering the risk for innovative products, an NPV of 49,138,429 CLP is obtained, so according to the established plans and considering only the sales in the national territory, this is a very attractive business. The expansion to the Latin American market in the third year and to Europe and the United States in the fourth year should be considered, which will make the business even more dynamic. (Capital Semilla, Chile), to promote the creation of a production plant or to finance a maquila process.

Table 4.34. *Consolidated investment costs for the preparation and start-up of the prototype.*

	Consolidated	
Activity	**Description**	**Pecuniary in thousands (CLP)**
Activity 1	Perform preliminary scale-up tests of the fermentation process.	2,670.00
Activity 2	Perform stability measurements for fermentation at different scales.	1,485.20
Activity 3	Determine times for fermentation at different scales.	1,580.50
Activity 4	Obtain laboratory scale formulation of prototype flavored beverages.	1,200.30
Activity 5	Perform sensory acceptance testing of the lab-scale flavored prototypes.	1,788.20
Activity 6	Determine the content of viable micro-organisms in the fermented beverages at different scales.	2,347.50
Activity 7	Determine the probiotic - immunobiotic capacities in the beverages at different scales.	2,492.70
Activity 8	Bottling tests and selection of packaging material.	3,457.10
Activity 9	Pilot testing of sensory acceptance of bottled beverages from different fermentation scales.	2,931.40
Activity 10	Determine the probiotic - immunobiotic capacities of the beverages after bottling for a shelf life of 30 days.	2,492.70
Activity 11	Trademark registration (INAPI) and SII.	3,650.00
USM	Scientific Production	3,904.40
HHRR	Administration, use of equipment, reagents, and laboratories	16,436,28
Totals		46,436.28
	Total, project	30,000,00
	Total, contribution	16,436,28

It will be necessary to continue with the marketing and commercial expansion plan to maintain a constant flow of customers through participation in events, fairs, seminars, congresses, etc., and the generation of alliances with public and private entities in the food and health sectors, both in Chile and in other

countries, taking advantage of the current contact networks and those obtained during the project. Resources may come from external investors interested in an alliance for the sale of this product, such as the company ALING, which markets panela-based products in Colombia; on the other hand, it can be estimated that resources may be obtained through government tenders, such as Corfo.

The costs and investments associated with the product testing and introduction phase are summarized in Table 4.33. There are also valuable resources provided by the university, such as bioreactors, HPLC, orbital shakers, laminar flow hoods, and a series of reagents and consumables that are valued according to their use and the amount of time and resources used. The average cost of an HPLC with the characteristics of the one at the San Joaquín campus of the UTFSM is approximately $45,984,538.58 CLP, and the costs associated with the service normally charged for the analysis of metabolites (ethanol, lactic acid, acetic acid, and sugars) in all the samples to be quantified in the project add up to approximately $6,121,260 CLP, given that the value per sample is 1UF ($28,339.17 CLP) and 216 determinations of said metabolites must be made.

The bioreactors available at the CBDAL to be used in this project have an approximate cost of 200,000,000 CLP, and considering the depreciation for annual use, estimated at 5% of the value of the equipment, the contribution can be summed up to 10,000,000 CLP. On the other hand, the use of the laminar flow hood, which has an approximate cost of $2,088,446, and estimating depreciation for use, the contribution would be $104,422 CLP. The orbital shakers to be used are 3 with a cost of $4,212,000 and a salvage value of $210,600 CLP. Therefore, the university will contribute not only access to the equipment but also its depreciated value for operating and administrative costs associated with the use of the laboratories and 3IE staff. In total, the university's contribution amounts to $16,436,282.

The project has a high economic and financial viability because the projections through the market study showed a positive NPV, also this product is promising due to its relative novelty, for this project to be a success story the most important and key factor is marketing and advertising to allow market penetration and massive awareness of the product.

Economic-social benefits or other potential impacts at the regional, national, or international level resulting from the use of the technology proposed in the project.

According to the latest census of 2017 by the National Statistics Institute (INE), the population of Chile is currently 17,574,003 people. Of these, 51% are women and 48.9% are men, of which the population segment of people between 15 and 64 years of age represents 80%. It is known that 50% of the Chilean adult population suffers from inflammatory bowel disease, so it could be said that approximately 7,025,126 people suffer from some kind of intestinal disease. In addition, it is worth remembering that, according to the Pan American Health Organization (PAHO), among the causes of death in adults between 65 and 79 years of age, 14.5% are due to diseases of the digestive system, and in those over 80 years of age, 10.6% (for the same reason).

This health problem may be strongly related to the intestinal microbiota present in sick people, which does not provide immunobiotic support or counteract pathogenic microorganisms at the intestinal level. Regular consumption of foods rich in viable probiotic microorganisms supports the restoration of gut flora and provides a defensive effect against disease. Yogurt drinks currently marketed as probiotics do not appear to have a major impact on gut health because the viable microorganism counts in these products at the time of consumption do not meet the FAO regulation, an indicator of whether the food has a real effect on the consumer. Many studies claim that microorganisms in these products lose their viability due to the long shelf life under refrigeration and the presence of fat. Yogurt as a probiotic food is also contraindicated for people with lactose intolerance, who need the consumption of these functional microorganisms to strengthen the intestinal flora that can metabolize various sugars and prevent colon inflation.

A probiotic yogurt drink can cost about $2,165 CLP per liter, which is not a high cost, but the purchase does not offer advantages over other higher-quality and more stable probiotics. Emerging kombucha beverages (a microbial community like kefir but made from tea) are a non-dairy potential probiotic alternative that is still under development, and the functional properties in vivo and in vitro of the microorganisms contained in these beverages are unknown. This product has the taste of acetic acid in its natural form, forcing manufacturers to heavily flavor and mask the taste, and the additives reduce the viability of potentially probiotic microorganisms that are undoubtedly present in their natural form. The kombucha that has penetrated the market the most is the Ghali brand, which costs $6.967 per liter; this would be the direct competitor to the beverage, which costs $3.750 per liter. Therefore, our product would be 46% cheaper and therefore more competitive. It is worth mentioning that the beverage has a standardized production process that, under certain conditions, can allow the viability of important

microorganisms to strengthen the immune system and naturally (without flavoring) has a pleasant taste. Prescription probiotics do their job, but these capsules often contain only one or two microbial species with limited function. The microbial community of water kefir acts symbiotically that could colonize the digestive tract with superiority. The average cost of these supplements is between $11,000 and $20,000 CLP (30 capsules), which means that a daily dose of this product would have an approximate value of $670 CLP.

Although it seems to be a reliable and economical alternative, buying all the doses can be unaffordable for many people who live on a daily income. On the other hand, the consumption of these supplements is not associated with a feeling of well-being and satisfaction, as is eating something delicious and nutritious; rather, they are directly related to the treatment of a disease and can be easily abandoned.

The main social impact is therefore to offer a beverage that contains what it claims to contain and helps people at risk of inflammatory bowel disease to improve their defenses and trigger the immune response in the gut, represented by the presence of anti-inflammatory interleukins in the epithelial cells of this organ. It also has an impact on people's diets, since the good taste of the drink allows it to accompany a wide variety of preparations, and this can help to replace the consumption of beverages with a high content of refined sugars and preservatives. The economic impact is also important, since our product is cheaper compared to the direct competition (kombucha) and could be approved as GRAS by prestigious research centers that have been supporting this project for more than 3 years.

The indirect benefits that the commercialization of this beverage can bring is the generation of employment, as we intend to support 3 people with or without technical skills in the first year, who will be trained in fermentation technology and basic microbiological analysis. It will also favor the chancaca and panela industries, providing the country with more income. In Colombia there is a situation with the panela industry, as the panela producers are generally farmers who want to improve their living conditions. It would be an important contribution to promote the purchase of their products and/or link them to the company as organized suppliers, the latter would be achieved by offering guild training so that their products are standardized, and their distribution chain is secure and stable. Panela in Colombia is highly valued, but its consumption is reduced to being a sweetener or to simply dilute it in hot water and drink it as an accompaniment to breakfast, other people drink this solution of panela with a little lemon and claim that it has a great energizing capacity, it is par excellence the drink of the great Colombian cycling champions in the world. For these reasons, panela is a product that, with a biotransformation such as the one we are generating, can have a much greater economic value in its transformations. This case is analogous to chancaca, as it is also known for its nutritional benefits.

Table 4.35. *Summary of planned activities and funding sources.*

Planned Activities	**Estimated timeframe in weeks Estimated total cost per Activity**	**Estimated timeframe in weeks Estimated total cost per Activity**	**Estimated timeframe in weeks Estimated total cost per Activity**	**Estimated timeframe in weeks Estimated total cost per Activity**
Carry out preliminary scale-up tests of the fermentation process.	6	2,670.00	2,670.00	
Perform stability measurements for fermentation at different scales.	10	1,485.20	1,485.20	
Determine times for fermentation at different scales.	15	1,580.50	1,580.50	
Obtain laboratory-scale formulation of prototype flavored beverages.	12	1,200.30	1,200.30	
Perform sensory acceptance tests of the flavored prototypes at laboratory scale.	3	1,788.20	1,788.20	
Determine the content of viable micro-organisms in the fermented beverages at different scales.	10	2,347.50	2,347.50	
Determine the probiotic - immunobiotic capacities of the beverages at different scales.	8	2,492.70	2,492.70	
Bottling tests and selection of packaging material.	2	3,457.10	3,457.10	
Pilot testing of sensory acceptance of bottled beverages from different fermentation scales.	3	2,931.40	2,931.40	
Determination of the probiotic-immunobiotic capacities of the beverages after bottling for a shelf life of 30 days.	20	2,492.70	2,492.70	
Trademark registration (INAPI) and at the SII.	8	3,650.00	3,650.00	
Scientific Production	16	3,904.40	3,904.40	
Administration, use of equipment, reagents, and laboratories.	25	16,436,28		16,436,28
Total		46,436.28	30,000.00	16,436,28

5. CONCLUSIONS

The evaluation of the stability of the microbial community showed that the imposition of different states to the fermentation can significantly intervene in the microbiological and physicochemical composition of the fermented beverages; therefore, the quality of fermented beverages can be affected when they are exposed to different substrates, different fermentation times or different agitation conditions. This is indicated because it has been observed that the composition of NCS varies according to its origin and the plant used for its production, and that violent agitation can cause changes that do not allow the stability and standardization of the production process. It appears that many changes in the fermentation process are barriers to obtaining consistently high quality beverages. This reinforces the idea of keeping the operating conditions under control, closing the system and how to better control the agitation at a higher level without causing situations that are difficult to manage.

It was possible to optimize the fermentation of non-centrifuged sugars with water kefir grains by obtaining response surface models for the different variables studied (ethanol, lactic acid, acetic acid, biomass, sugar consumption, abundance of major microbial groups), where an operating point was obtained that responds to the constraints imposed to obtain a beverage of pleasant sensory quality (physicochemical and microbiological quality), stabilized and standardized. The fact that this fermentation could be optimized in this study, and had not been done in any other study, suggests that the conditions evaluated in this work are novel and that, due to their high statistical reproducibility, they can be applied to other studies using WKGs to produce standard beverages with different substrates or with different sensory quality objectives.

Strains of microorganisms were obtained through dependent and independent culture techniques in proportions typical of fermentations carried out with water kefir grains observed worldwide, indicating that these grains have a common origin and that their microbiological variability is due to adaptation to different substrates. This can be inferred from the presence of Liquorilactobacillus nagelii, a microorganism that has not been widely reported in the grain as such but is associated with its fermentation in uncentrifuged sugar molasses and is speculated to be a new species in this microbial community.

The proposal of an economic feasibility project for the sale of this fermented beverage allowed to understand how, at the market level, it is possible to penetrate different target groups, such as the elderly over 65 years of age and school children, according to the fact that these are the populations with the greatest need for reinforcement of the immune and digestive systems and that in these beverages,

although their probiotic capacity has not yet been proven, they have a high promising functional potential due to the microorganisms and metabolites they contain.

The study of the optimal operating conditions for the formation of metabolites and, therefore, for the development of the flavor of water-kefir fermented beverages can allow the design of different beverages aimed at different target groups, since imposing different restrictions on the software and directing the mathematical optimization to what is desired can help to obtain beverages that are higher in alcohol, more acidic or, on the contrary, much milder. It is important to note that the biogenesis of large microbial groups occurs under conditions that are opposed to the formation of metabolites; this also indicates that the operating conditions can be modulated in such a way as to obtain beverages with a high microbial load or, conversely, with a much higher proportion of metabolites.

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6. RECOMMENDATIONS AND PROJECTIONS

Little or nothing is known about the probiotic power of non-centrifuged sugar fermented beverages with water kefir granules; therefore, it is proposed to study the microbial strains isolated from grains from Latin America (strains obtained in this study) to determine their probiotic capacities and possibly enrich the grains and beverages obtained with these strains. The MALDI-TOF study also revealed the presence of many unidentified microorganisms that can be studied to discover new microbial species in kefir granules with novel functional and technological properties.

In this study, it was observed that agitation can interfere with the fermentation of water kefir grains because the grains are very irregular, fragile due to the nature of dextran, and can be up to 3 cm in diameter. If the size of the reactor in which they are placed for fermentation is very small, it may happen that, although the fermentation system is agitated, the grains move very little, and the agitation interferes little or not at all with the exchange of substrate. But if the pellet manages to float freely in 500 mL or larger reactors, with geometries where the reactor neck size is larger than the pellet size, and without being highly agglomerated, agitation can interfere with substrate exchange, which would affect sugar consumption, biogenesis, and metabolite formation, and this will be very unpredictable. All the above is explained to leave as a projection that exogenous factors such as agitation (a higher order property) should continue to be studied in this type of fermentation to give predictive models where agitation is better adapted to mathematical models and thus be able to perform safe and controlled scale-ups.

Exogenous control variables (higher order properties) such as reactor geometry, mechanical stirring with paddles or air, and oxygen exchange with the external medium must continue to be studied in order to better understand the phenomena of fermentation with water kefir granules and thus provide estimated response variables with better settings and be able to establish a more controlled process that allows obtaining beverages of a more standardized and stable character at larger scales.

The WKG is adaptable to different sugary substrates, such as extracts and fruits; it could also ferment milk, soy, and other types of vegetables and nuts. Given this versatility, this microbial community is potentially a functional starter culture that can add value to different raw materials and is projected as a non-dairy probiotic food that can benefit a wide audience with gastrointestinal issues. Unrefined sugar is a very economical raw material with high nutritional value, providing a substrate that promotes the growth of kefir grains due to micronutrient content. In addition, the use of NCS in the production of non-dairy probiotic beverages could be a significant market opportunity, benefiting the agro-industrial sector of Latin American countries and offering the possibility of diversifying this food. The non-centrifuged

sugar cane enhances the grain growth in contrast to the German substrate (lemon slices, figs, and refined sugar).

Given the ability of water kefir grains to transform various foods, the projection and pursuit of developing products and processes based on fermentation with this microbial community should be considered. Its high nutritional value, low cost, and versatility make WKG an ideal crop for the standardization of fermented food processes, such as alcoholic and non-alcoholic beverages, vinegars, sauerkraut, pickles, cheeses, and other potential products that do not yet exist in the market.

In water kefir grains, it has been observed that, despite the diversity of their origin, adaptation to different substrates, manipulation, and state of activity, there are commonalities in terms of metabolite content and biomass yields. Therefore, it is recommended to use the empirical equations found in this study as references for other similar processes carried out with water kefir grains.

It is proposed to include the genera *Oenococcus* and *Gluconobacter* in the definitions of microbiological composition, as these microorganisms have been recently discovered in studies conducted over the last three years on water kefir grains. It is important to investigate their role in this community more thoroughly and to uncover their technological and functional properties. Additionally, it is recommended to study the probiotic properties of the lactic acid bacteria in this grain to provide a definition that allows kefir to be recognized as a health-beneficial beverage for consumers.

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8. APPENDIX

8.1. APPENDIX: Methodology strategy

Table A. *The following table describes the methodological strategy for the development of this study in 5 main stages.*

<table>
<tr><th colspan="3">Research Strategy Stages</th></tr>
<tr><td colspan="3">Stage 1</td></tr>
<tr><td colspan="3">Selection of Water Kefir Grains (WKG) from different origins</td></tr>
<tr><td colspan="3">Experimental Design</td></tr>
<tr><td rowspan="6">5 Different grains of WKGs (3 from Colombia and 2 from Chile)</td><td rowspan="6">2 Different Water Kefir Grains</td><td>Sensory and regulatory criteria for the selection of WKGs</td></tr>
<tr><td>Lactic Acid (1.2 - 3) g/L</td></tr>
<tr><td>Acetic acid ~ 0 g/L - (As low as possible)</td></tr>
<tr><td>Ethanol <10g/L</td></tr>
<tr><td>Biomass (The best possible increase)</td></tr>
<tr><td>Sugars <50 g/L</td></tr>
<tr><td colspan="3">Stage 2</td></tr>
<tr><td colspan="3">Identification and determination of the relative abundance of microbial populations in the 2 selected WKGs</td></tr>
<tr><td colspan="3">Preliminary identification and relative abundance of species in selected WKGs and stability analysis according to substrate and temperature change at final fermentation time (96 hours).</td></tr>
<tr><td colspan="3">Experimental Design 2^3 Factorial</td></tr>
<tr><td rowspan="4">WKG1 (Selected)</td><td rowspan="2">Cane Sugar (Panela)</td><td>Temperature 1 (21°C)</td></tr>
<tr><td>Temperature 2 (37°C)</td></tr>
<tr><td rowspan="2">Beet Sugar (Chancaca)</td><td>Temperature 1 (21°C)</td></tr>
<tr><td>Temperature 2 (37°C)</td></tr>
<tr><td rowspan="4">WKG2 (Selected)</td><td rowspan="2">Cane Sugar (Panela)</td><td>Temperature 1 (21°C)</td></tr>
<tr><td>Temperature 2 (37°C)</td></tr>
<tr><td rowspan="2">Beet Sugar (Chancaca)</td><td>Temperature 1 (21°C)</td></tr>
<tr><td>Temperature 2 (37°C)</td></tr>
<tr><td colspan="3">Stage 3</td></tr>
<tr><td colspan="3">Determination of optimal operating points using Response Surface Methodology (RSM) optimization and definitive identification and determination of the relative abundance of microbial species and analysis of changes in these populations in the WKGs at 48 and 96 hours using Standard Deviation (SD) function analysis.</td></tr>
<tr><td colspan="3">Experimental Design Central Composite Design (CCD), 8 axial points and 6 replicates at the central point, with α = 1.414</td></tr>
<tr><td rowspan="6">For each WKG (Selected)</td><td>CCD Design</td><td>Sensory and regulatory criteria for the Optimization of WKGs fermentation</td></tr>
<tr><td rowspan="2">Factors to control</td><td>Temperature (20 - 40°C)</td></tr>
<tr><td>Agitation (0 - 160 rpm)</td></tr>
<tr><td rowspan="2">Response variables</td><td>Lactic Acid Bacteria (Maximize)</td></tr>
<tr><td>Acetic acid Bacteria (Minimize)</td></tr>
</table>

		Yeast (Minimize)
		Lactic Acid (1.2 - 3) g/L
		Acetic acid ~ 0 g/L - (As low as possible)
		Ethanol <10g/L
		Biomass (The best possible increase)
		Glucose (Maximun consumption) <50g/L
		Fructose (Maximun consumption) <50g/L
		Sucrose (Maximun consumption) <50g/L

Stage 4

Analysis of biomass production when substrate is changed in grains from Latin America (Colombia and Chile—selected WKGs) and Europe (Germany) and identification of microorganisms using culture-dependent techniques.

Experimental Design

WKG (Origin)	**Conventional Substrate (NCS or refined sugar)**	**New substrate (replace NCS substrate with refined sugar and vice versa)**	**Measurements**
Germany (conventional substrate-refined sugar)	2 fermentation steps	6 fermentation steps	Biomass (The best possible increase) (Gravimetric)
Colombian WKG (conventional substrate-NCS)			pH (Degree of Acidification)
Chilean WKG (conventional substrate-NCS)			Microorganism Cultivation, MALDI-TOF and 16s DNA Sequencing

Stage 5

Proposed project for the development of the NCS fermented beverage with water kefir grains, based on the results obtained in this thesis and through a technical-economic feasibility studyProposed project for the development of the NCS fermented beverage with water kefir grains, based on the results obtained in this thesis and through a technical-economic feasibility study

Method

Activity 1: Following the optimal operating point found in this research, validate it at volumes up to 10 liters.

Activity 2: Understand microbial kinetics to determine operating time at larger process volumes.

Activity 3: Levels of viable microorganisms in the final product.

Activity 4: Probiotic abilities of microorganisms.

Activity 5: Obtain prototypes of flavored and natural beverages using traditional fruits and plants from our regions.

Activity 6: Sensory profile of prototype beverages.

Activity 7: Packaging tests in containers made of different materials with sustainable or reusable characteristics. Determination of usable volume and use of headspace gases. Stability tests during shelf life.

Activity 8: Sensory tests with users at strategic points according to market analysis.

Activity 9: Patentability study, evaluation of intellectual property strategy, and registration of trademarks and trade secrets.

Activity 10: Formalization of the legal entity.

8.2. APPENDIX: Calculations Equations

- **Equation A.** Sugar Consumption calculation (%).

$$\textbf{Sugar Consumption}\ (\%) = \left(\frac{\textbf{Sugar}_{\textit{Initial}}\ \left(\frac{g}{L}\right) - \textbf{Sugar}_{\textit{Final}}\ \left(\frac{g}{L}\right)}{\textbf{Sugar}_{\textit{Initial}}\ \left(\frac{g}{L}\right)}\right) * \mathbf{100}$$

- **Equation B.** Calculation of the titratable acid content expressed in terms of lactic acid.

$$\textbf{Titratable Acidity}\ (g/L) = \left(\frac{(\textbf{NaOH volume spent}\ (L) * \textbf{NaOH Normality}\ \left(\frac{mol}{L}\right) * \textbf{Lactic Acid molecular weight}\ \left(\frac{g}{mol}\right))}{\textbf{Aliquot Volume}\ (L)}\right) * \mathbf{100}$$

Where: NaOH is the Sodium hydroxide.

- **Equation C.** Water Kefir Grains (WKG) Biomass increase calculation (%).

$$\textbf{WKG Biomass increase}\ (\%) = \left(\frac{\textbf{Biomass}_{\textit{Initial}}\ (g/L) - \textbf{Biomass}_{\textit{Final}}\ (g/l)}{\textbf{Biomass}_{\textit{Final}}\ (g/l)}\right) * \mathbf{100}$$

- **Equation D.** Relative abundance calculation

$$\textbf{Relative Abundance}\ (\%) = \left(\frac{\textbf{Individual sequences}\ * \mathbf{100}}{\textbf{Total secuences}}\right)$$

8.3. APPENDIX: Templates used for sensory acceptance testing (sensory profile.)

Figure A: Template for testing the sensory acceptance profile of fermented WKGCOL3 and WKGCH2.

EVALUACIÓN SENSORIAL

FORMATO PERFIL DE SABOR

NOMBRE:_____FECHA_____

NOMBRE DEL PRODUCTO_____

Frente a usted hay una muestra de kéfir de agua, la cual debe probar, describiendo las características de sabor que estén presentes.

Marque con una X sobre la casilla del término que más describa lo que usted siente en la muestra.

SABOR	0	1	2	3	4	5
Dulce						
Acido						
Amargo						
Fermentado						
Afrutado						
Astringente						
Picante						
Metálico						

COMENTARIOS:

_____

_____

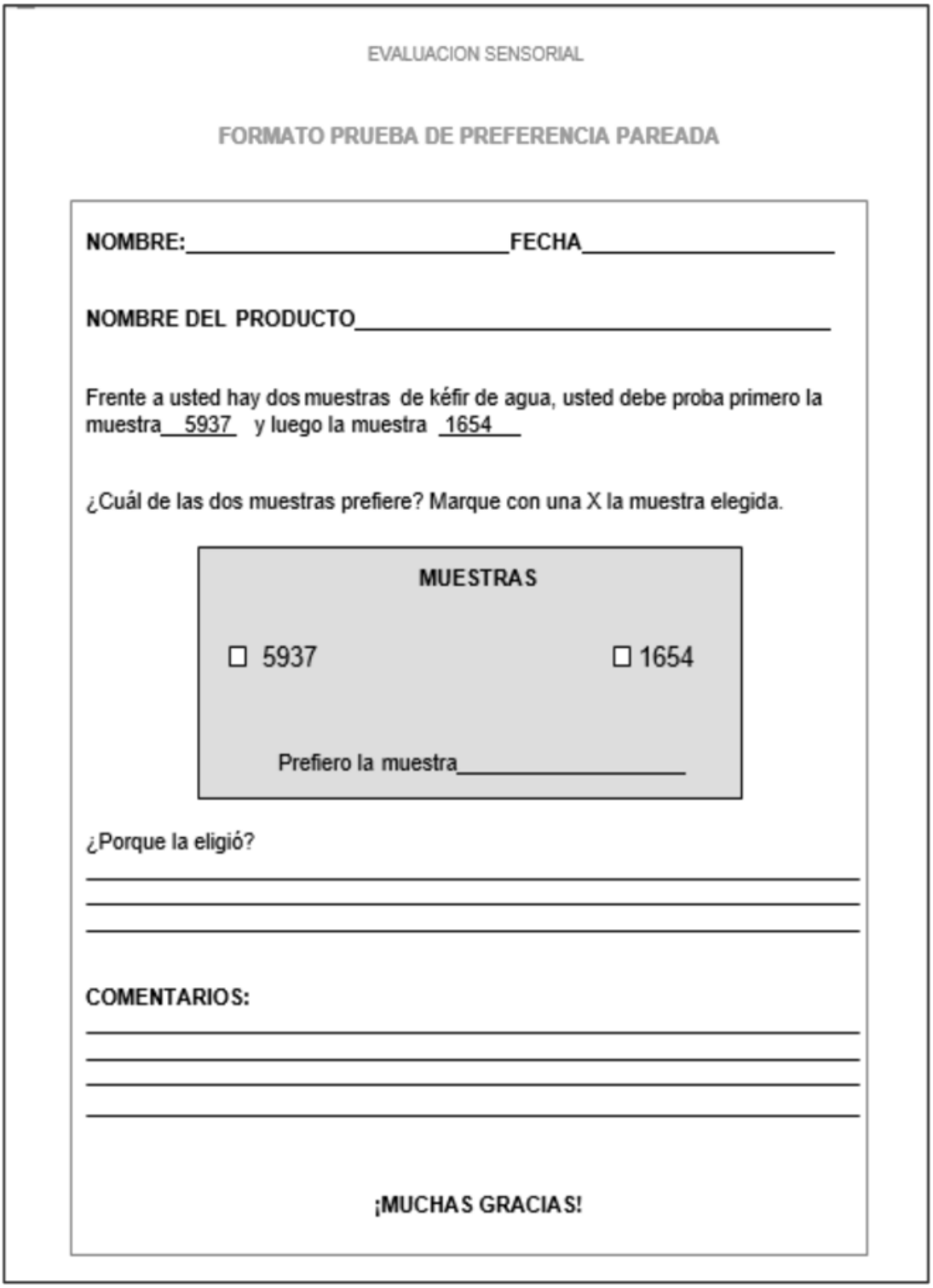
_____

_____

_____

¡MUCHAS GRACIAS!

Figure B: Templates for Sensory Acceptance Preference Testing of WKGCOL3 and WKGCH2 Fermented beverage.



EVALUACION SENSORIAL

FORMATO PRUEBA DE PREFERENCIA PAREADA

NOMBRE:_____FECHA_____

NOMBRE DEL PRODUCTO_____

Frente a usted hay dos muestras de kéfir de agua, usted debe proba primero la muestra 5937 y luego la muestra 1654

¿Cuál de las dos muestras prefiere? Marque con una X la muestra elegida.

MUESTRAS

☐ 5937 ☐ 1654

Prefiero la muestra_____

¿Porque la eligió?

_____

_____

_____

COMENTARIOS:

_____

_____

_____

_____

¡MUCHAS GRACIAS!

8.4. APPENDIX: Preliminary metabarcoding analysis

Table A. *260/280 ratio measure of quality of extracted DNA for samples (WKGCOL3 and WKGCH2) according to preliminary study.*

Grain code	ng/uL	260/280
COLPAN21	2.8	2.18
COLPAN37	2.1	2.12
COLCH21	2.9	1.81
COLCH37	2.2	1.32
CHPAN21	2.8	2.97
CHPAN37	5.9	1.78
CHCH21	2.6	1.4
CHCH37	2.5	1.37

Table B. *The standard deviations (SD) and arithmetic means (X) are shown, calculated generally for each species and genera of bacteria across all treatments and among the experiments where temperature (°C) or substrate (S) was also varied for each species and genus.*

Colombian Water Kefir Grain (WKGCOL3)										
Genus	***Mean (X) General***	***SD General***	***Mean (X) Panela***	***SD (T°C) Panela***	***Mean (X) Chancaca***	***SD (T°C) Chancaca***	***Mean 37°C***	***SD (S) 37°C***	***Mean 21°C***	***SD (S) 21°C***
Lactobacillus	***92,51***	2,54	93,16	2,81	91,86	3,12	94,6	0,76	90,41	1,07
Acetobacter	1,74	2,4	0,62	0,83	2,86	3,41	0,24	0,3	3,24	2,87
Gluconobacter	1,01	1,12	0,3	0,36	1,71	1,29	0,42	0,53	1,59	1,46
Oenococcus	1,43	0,57	1,68	0,66	1,18	0,53	1,39	0,24	1,48	0,95
Species	***Mean (X) General***	***SD General***	***Mean (X) Panela***	***SD (T°C) Panela***	***Mean Chancaca***	***SD (T°C) Chancaca***	***Mean 37°C***	***SD (S) 37°C***	***Mean 21°C***	***SD (S) 21°C***
Lactobacillus ghanensis	44,19	4,24	43,66	4,83	44,72	5,43	43,98	4,38	44,4	5,88
Lactobacillus_casei/ paracasei	16,26	3,24	16,86	5,2	15,67	1,75	15,05	2,64	17,48	4,31
Lactobacillus harbinensis/perolens	11,9	4,08	12,85	3,32	10,96	5,93	15,18	0,04	8,63	2,64
Lactobacillus hilgardii	9,92	4,03	8,1	4,15	11,75	4,26	6,95	2,52	12,9	2,64
Lactobacillus diolivorans	7,81	3,64	8,29	4,5	7,34	4,32	10,93	0,76	4,69	0,58
Lactobacillus parabuchneri	3,78	0,88	4,47	0,28	3,1	0,62	3,91	0,52	3,66	1,41
Lactobacillus satsumensis	0,01	0,01	0,01	0	0,02	0,02	0,01	0,01	0,02	0,01
Lactobacillus perolens	0,02	0,01	0,02	0,01	0,02	0,01	0,01	0	0,03	0
Oenococcus alcoholitolerans	0,82	0,64	1,16	0,88	0,49	0,12	0,47	0,09	1,18	0,86
Oenococcus oeni	0,67	0,41	0,59	0,2	0,74	0,66	0,97	0,34	0,36	0,13

Continuation Table B, Appendix 8.4

Chilean Water Kefir Grain (WKGCH2)										
Genus	***Mean (X) General***	***SD General***	***Mean (X) Panela***	***SD (T°C) Panela***	***Mean Chancaca***	***SD (T°C) Chancaca***	***Mean 37°C***	***SD (S) 37°C***	***Mean 21°C***	***SD (S) 21°C***
Lactobacillus	92,59	3,93	92,92	2,22	92,25	6,4	95,63	1,61	89,54	2,56
Acetobacter	2,55	2,68	2,07	2,64	3,03	3,7	0,31	0,15	4,79	1,21
Gluconobacter	1,05	1,31	0,39	0,43	1,72	1,8	0,27	0,25	1,84	1,63
Oenococcus	0,36	0,26	0,41	0,03	0,31	0,44	0,51	0,17	0,22	0,31
Species	***Mean (X) General***	***SD General***	***Mean (X) Panela***	***SD (T°C) Panela***	***Mean Chancaca***	***SD (T°C) Chancaca***	***Mean 37°C***	***SD (S) 37°C***	***Mean 21°C***	***SD (S) 21°C***
Lactobacillus ghanensis	37,64	5,82	42,3	1,51	32,98	3,54	39,42	5,57	35,85	7,61
Lactobacillus_casei/ paracasei	17,88	2,14	18,91	0,69	16,85	3	16,58	2,61	19,19	0,3
Lactobacillus harbinensis/perolens	18,35	4,56	14,92	0,47	21,78	3,9	19,9	6,57	16,81	3,13
Lactobacillus hilgardii	9,92	2,38	8,09	0,01	11,76	1,87	10,59	3,52	9,26	1,66
Lactobacillus diolivorans	4,56	1,14	4,67	1,71	4,45	0,96	5,51	0,53	3,62	0,22
Lactobacillus parabuchneri	4,22	0,8	3,55	0,04	4,89	0,37	4,07	0,78	4,37	1,11
Lactobacillus satsumensis	1,71	0,87	2,13	0,7	1,29	1,03	1,1	0,76	2,32	0,42
Lactobacillus_perolens	0,48	0,11	0,39	0,03	0,57	0,07	0,45	0,11	0,52	0,15
Oenococcus alcoholitolerans	0,48	0,11	0,44	0,05	0,52	0,16	0,44	0,05	0,52	0,16
Oenococcus oeni	0,03	0,02	0,02	0,01	0,04	0,02	0,04	0,02	0,02	0,01

Table C. *The standard deviations (SD) and arithmetic means (X) are shown, calculated generally for each species and genus of bacteria across all treatments and among the experiments where temperature (°C) or substrate (S) was also varied for each species and genus.*

Colombian Water Kefir Grain (WKGCOL3)										
Genus	***Mean (X) General***	***SD General***	***Mean (X) Panela***	***SD (T°C) Panela***	***Mean Chancaca***	***SD (T°C) Chancaca***	***Mean 37°C***	***SD (S) 37°C***	***Mean 21°C***	***SD (S) 21°C***
Zygotorulaspora	18,4	19,96	20,13	27,37	16,66	20,82	1,36	0,83	35,43	5,73
Schizosaccharomyces	1,66	0,51	1,82	0,48	1,49	0,67	2,06	0,14	1,25	0,33
Saccharomyces	31,22	6,11	31,91	5,33	30,53	9,03	32,53	6,21	29,91	8,15
Dekkera	48,28	20,17	45,97	32,46	50,58	12,1	64,03	6,92	32,52	13,44
Candida	0,45	0,67	0,16	0,23	0,74	0,98	0,02	0,03	0,88	0,78
Species	***Mean (X) General***	***SD General***	***Mean (X) Panela***	***SD (T°C) Panela***	***Mean Chancaca***	***SD (T°C) Chancaca***	***Mean 37°C***	***SD (S) 37°C***	***Mean 21°C***	***SD (S) 21°C***
Zygotorulaspora florentina	18,4	19,96	20,13	27,37	16,66	20,82	1,36	0,83	35,43	5,73
Schizosaccharomyces pombe	1,66	0,51	1,82	0,48	1,49	0,67	2,06	0,14	1,25	0,33
Saccharomycodes ludwigii	2,4	0,98	2,2	1,29	2,6	1,03	1,58	0,41	3,22	0,15
Saccharomyces cerevisiae	28,82	6,34	29,7	4,05	27,94	10,06	30,95	5,8	26,69	8,3
Dekkera bruxellensis	48,28	20,17	45,97	32,46	50,58	12,1	64,03	6,92	32,52	13,44
Candida californica	0,45	0,67	0,16	0,23	0,74	0,98	0,02	0,03	0,88	0,78

Continuation Table C, Appendix 8.4

Chilean Water Kefir Grain (WKGCH2)										
Genus	***Mean (X) General***	***SD General***	***Mean (X) Panela***	***SD (T°C) Panela***	***Mean Chancaca***	***SD (T°C) Chancaca***	***Mean 37°C***	***SD (S) 37°C***	***Mean 21°C***	***SD (S) 21°C***
Zygotorulaspora	48,52	35,48	50,62	30,95	46,41	52,92	18,87	13,96	78,17	8,01
Saccharomyces	19,49	15,6	24,09	21,95	14,9	12,82	31,79	11,06	7,2	1,93
Dekkera	31,69	25,19	25,04	9,29	38,34	40,51	49,3	25,02	14,08	6,2
Candida	0,3	0,29	0,25	0,29	0,34	0,4	0,05	0,01	0,54	0,13
Species	***Mean (X) General***	***SD General***	***Mean (X) Panela***	***SD (T°C) Panela***	***Mean Chancaca***	***SD (T°C) Chancaca***	***Mean 37°C***	***SD (S) 37°C***	***Mean 21°C***	***SD (S) 21°C***
Zygotorulaspora florentina	48,52	35,48	50,62	30,95	46,41	52,92	18,87	13,96	78,17	8,01
Saccharomycodes ludwigii	0,18	0,14	0,24	0,2	0,12	0,07	0,28	0,15	0,09	0,02
Saccharomyces cerevisiae	19,31	15,47	23,85	21,75	14,78	12,75	31,51	10,92	7,11	1,91
Dekkera bruxellensis	31,69	25,19	25,04	9,29	38,34	40,51	49,3	25,02	14,08	6,2
Candida californica	0,3	0,29	0,25	0,29	0,34	0,4	0,05	0,01	0,54	0,13

8.5. APPENDIX: Water Kefir Grain Selection Criteria and Metabolic Optimization at 500 and 250 mL.

Table A. *Scoring for each evaluated parameter according to the established criteria (biomass increase, acidity, ethanol content, and sugar consumption).*

Kefir Grains code	Biomass increase (%)	Acidity (g/L)	Ethanol (g/L)	Sugar Consumption (%)	Total Score
WKGCOL	1.0	0.0	0.0	1.0	2.0
WKGCOL2	1.0	1.0	0.0	0.0	2.0
WKGCOL3	2.0	1.0	1.0	1.0	5.0
WKGCH	0.0	1.0	1.0	2.0	4.0
WKGCH2	2.0	2.0	2.0	1.0	7.0

Table B. *The ANOVA table shows the test statistics for the response variables (FG, LA, AA, EtOH, and Sugars) with quadratic adjustment or two-term interaction for WKGCOL3 and WKGCH2, respectively, in the experiments conducted at 500 mL.*

Source	***Df***	***Final grain (FG)***	***Latic acid (LA)***	***Acetic acid (AA)***	***Ethanol (EtOH)***	***Glucose (GLUC)***	***Fructose (FRUC)***	***Sucrose (SUCR)***
		p-value	***p-value***	***p-value***	***p-value***	***p-value***	***p-value***	***p-value***
Regression Equation WKGCOL3	*5*	*0,0179*	*0,0012*	*0,3703*	*0,4364*	*0,5767*	*0,2795*	*0,3883*
X_1-Agitation (RPM)	*1*	*0,1484*	*0,5057*	*0,8631*	*0,4684*	*0,6766*	*0,2707*	*0,4922*
X_2-Temperature (°C)	*1*	*0,0155*	*0,0001*	*0,964*	*0,1438*	*0,516*	*0,5452*	*0,219*
X_1X_2	*1*	*0,2052*	*0,1113*	*0,45*	*0,2683*	*0,1342*	*0,3026*	*0,2025*
X_1^2	*1*	*0,016*	*0,0847*	*0,0489*	*0,9048*	*0,5057*	*0,734*	*0,4818*
X_2^2	*1*	*0,1127*	*0,0149*	*0,7942*	*0,3978*	*0,7078*	*0,0632*	*0,2768*
				Statistical parameters				
R^2		*0,7726*	*0,8884*	*0,4385*	*0,4039*	*0,3347*	*0,4903*	*0,4289*
R^2 adj		*0,6305*	*0,8187*	*0,0876*	*0,0314*	*-0,0812*	*0,1718*	*0,072*
Pred-R^2		*-0,596*	*0,2168*	*-25,912*	*-32,209*	*-35,746*	*-26,088*	*-29,298*
Adeq Precision		*8,984*	*10,602*	*30,828*	*35,677*	*32,794*	*34,145*	*3,561*
CV. %		*8,41*	*0,908*	*0,1574*	*4,41*	*2,54*	*1,77*	*14,08*
Source	***Df***	***Final grain (FG)***	***Latic acid (LA)***	***Acetic acid (AA)***	***Ethanol (EtOH)***	***Glucose (GLUC)***	***Fructose (FRUC)***	***Sucrose (SUCR)***
		p-value	***p-value***	***p-value***	***p-value***	***p-value***	***p-value***	***p-value***
Regression Equation WKGCH2	*5*	*0,3259*	*0,0065*	*0,5905*	*0,5263*	*0,3681*	*0,126*	*0,0461*
X_1-Agitation (RPM)	*1*	*0,4883*	*0,7963*	*0,595*	*0,7927*	*0,9778*	*0,4125*	*0,7193*
X_2-Temperature (°C)	*1*	*0,2768*	*0,0007*	*0,5761*	*0,1493*	*0,1337*	*0,513*	*0,0202*
X_1X_2	*1*	*0,4266*	*0,2644*	*0,3357*	*0,396*	*0,1681*	*0,9767*	*0,0815*
X_1^2	*1*	*0,2781*	*0,0352*	*0,7622*	*0,3415*	*0,3061*	*0,8976*	
X_2^2	*1*	*0,143*	*0,175*	*0,179*	*0,9071*	*0,9041*	*0,0107*	
				Statistical parameters				
R^2		*0,4631*	*0,8269*	*0,328*	*0,3592*	*0,4397*	*0,6037*	*0,5348*
R^2 adj		*0,1275*	*0,7188*	*-0,0921*	*-0,0412*	*0,0896*	*0,356*	*0,3953*
Pred-R^2		*-0,5348*	*-0,2243*	*-34,147*	*-34,734*	*-29,107*	*-2*	*-0,8366*
Adeq Precision		*45,805*	*83,345*	*24,928*	*32,542*	*41,174*	*47,546*	*72,728*
CV. %		*11,39*	*25,63*	*33,67*	*28,94*	*25*	*15,09*	*27,074*

Table C. *Constraints used for the WKGCOL3 and WKGCH2 fermentations, where the number of + symbols indicate the level of importance used. The goal for each variable is to maximize, minimize, or keep it within a range to give, take, or keep it a weight for optimization purposes.*

Response	Goal	Limits	Importance
FG (g/L)	in range	88.97 - 133.48	-
LA (g/L)	in range	1.24 - 11.34	-
AA (g/L)	minimize	0.13 - 1.16	++++
EtOH (g/L)	minimize	6.21 -23.42	+++
GLUC (g/L)	in range	2.83 -10.7	-
FRUC (g/L)	in range	3.99 - 19.67	-
SUCR (g/L)	minimize	7.16 - 36.72	+++

Table D. *The standard deviations (SD) are observed for the data sets of each WKG under the same operating conditions; the data used corresponds to the experiments conducted at 250 and 500 mL.*

run		***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9, 10, 11, 12, 13, 14***
Agitation (rpm)		*136,57*	*160*	*136,57*	*80*	*23,43*	*0*	*23,43*	*80*	*80*
Temperature (°C)		*22,93*	*30*	*37,07*	*40*	*37,07*	*30*	*22,93*	*20*	*30*
FG (SD)	***WKGCOL3***	*2,86*	*9,31*	*1,65*	*1,88*	*7,72*	*10,47*	*21,3*	*1,62*	*3,9*
	WKGCH2	*16,31*	*13,18*	*1,91*	*1,21*	*2,6*	*7,57*	*2,09*	*7,36*	*9,6*
	General	*10,61*	*14,17*	*4,67*	*3,76*	*7,8*	*10,08*	*14,62*	*6,93*	*8,48*
LA (SD)	***WKGCOL3***	*0,59*	*1,12*	*1,44*	*0,74*	*1,65*	*0,56*	*0,88*	*0,04*	*0,86*
	WKGCH2	*0,94*	*1,2*	*0,18*	*0,18*	*1,03*	*1,6*	*1,82*	*0,15*	*0,5*
	General	*0,64*	*1,18*	*0,9*	*0,44*	*1,64*	*1,14*	*1,19*	*0,09*	*0,72*
AA (SD)	***WKGCOL3***	*0,13*	*0,08*	*0,18*	*0,01*	*0,18*	*0,08*	*0,25*	*0,1*	*0,15*
	WKGCH2	*0,12*	*0,23*	*0,18*	*0,04*	*0,06*	*0,11*	*0,12*	*0,05*	*0,07*
	General	*0,22*	*0,22*	*0,2*	*0,1*	*0,15*	*0,17*	*0,24*	*0,11*	*0,17*
EtOH (SD)	***WKGCOL3***	*0,1*	*8,08*	*4,19*	*0,84*	*2,32*	*7,06*	*1,21*	*1,56*	*1,26*
	WKGCH2	*0,88*	*8,53*	*1,29*	*1,2*	*1,05*	*7,27*	*3,36*	*0,44*	*0,52*
	General	*1,84*	*6,82*	*2,9*	*1,32*	*1,49*	*5,9*	*2,52*	*1,04*	*0,95*
GLUC (SD)	***WKGCOL3***	*0,29*	*3,48*	*1,36*	*0,71*	*2,14*	*3,35*	*2,64*	*0,67*	*0,88*
	WKGCH2	*0,62*	*1,97*	*0,28*	*2,03*	*0,98*	*2,54*	*2,38*	*0,59*	*0,4*
	General	*1,04*	*2,31*	*1,26*	*1,28*	*1,36*	*2,46*	*2,15*	*0,87*	*0,82*
FRUC (SD)	***WKGCOL3***	*1,15*	*1,42*	*3,23*	*5,81*	*4,04*	*0,86*	*5,29*	*2,57*	*2,6*
	WKGCH2	*1,23*	*1,64*	*1,24*	*4,88*	*1,75*	*0,47*	*2,2*	*0,45*	*1,79*
	General	*1,79*	*1,56*	*2,78*	*5,06*	*3,26*	*2,72*	*4,27*	*2,37*	*2,79*
SUCR (SD)	***WKGCOL3***	*0,93*	*21,45*	*1,28*	*8,23*	*19,23*	*24,02*	*3,15*	*18,51*	*13,02*
	WKGCH2	*4,41*	*14,96*	*1,62*	*2,14*	*10,45*	*15*	*5,89*	*6,79*	*7,21*
	General	*4,4*	*15,12*	*2,08*	*6,38*	*13,14*	*16,75*	*3,86*	*11,75*	*10,54*

Table E. *The ANOVA table shows the test statistics for the response variables (FG, LA, AA, EtOH, and Sugars) with quadratic adjustment or two-term interaction for WKGCOL3 and WKGCH2, respectively, in the experiments conducted at 250 mL.*

Source	**df**	**Final grain**	**Latic acid**	**Acetic acid**	**Ethanol**	**Glucose**	**Fructose**	**Sucrose**
WKGCOL3		**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**
Empirical Model	5	0.0055	0.0002	0.0142	< 0.0001	0.0003	0.0024	0.0533
X_1-RPM	1	0.2814	0.1846	0.1239	0.1541	0.1728	0.029	0.9854
X_2-T	1	0.0011	< 0.0001	0.5226	0.0003	0.0017	0.0267	0.1414
X_1X_2	1	0.8552		0.7715	0.8829	0.5772	0.0621	0.7073
X_1^2	1	0.0095		0.406	< 0.0001	0.0002	0.0076	0.0737
X_2^2	1	0.215		0.001	0.0085	0.0007	0.0028	0.0139
Statistical parameters for the model								
R^2		0.8339	0.7802	0.7865	0.9423	0.9248	0.8671	0.9510
R^2 adj		0.7302	0.7402	0.653	0.9063	0.8778	0.784	0.9204
Pred-R^2		0.1299	0.5253	0.4991	0.6026	0.4709	0.240	0.6521
Adeq Precision		9.8400	13.147	65.412	19.383	15.285	14.95	70.987
CV. %		3.480	26.910	17.660	5.400	11.30	12.56	19.413
Source	**df**	**Final grain**	**Latic acid**	**Acetic acid**	**Ethanol**	**Glucose**	**Fructose**	**Sucrose**
WKGCH2		**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**
Empirical Model	5	0.0022	< 0.0001	0.0142	< 0.0001	0.0033	0.0112	< 0.0001
X_1-RPM	1	0.8254	0.2317	0.1239	0.0302	0.5908	0.0686	0.2859
X_2-T	1	0.0006	< 0.0001	0.5226	< 0.0001	0.0206	0.0187	< 0.0001
X_1X_2	1			0.7715	0.9048	0.9749	0.3458	0.2608
X_1^2	1			0.406	< 0.0001	0.0009	0.0171	0.0219
X_2^2	1			0.001	0.0003	0.0141	0.0254	0.0795
Statistical parameters for the model								
R^2		0.6722	0.8176	0.909	0.9642	0.8551	0.7996	0.957
R^2 adj		0.6126	0.7844	0.8522	0.9419	0.7646	0.6744	0.9301
Pred-R^2		0.3448	0.6109	0.4896	0.7498	-0.0146	-0.4214	0.7028
Adeq Precision		10.248	14.921	15.804	25.828	11.891	10.32	19.363
CV. %		5.310	25.240	9.650	5.310	12.440	15.390	10.890

Table F. *Summary table showing the data for the maximum and minimum amounts of metabolites (LA, AA, EtOH) and for the major microbial groups obtained and their respective experimental conditions.*

Volume (mL)	*Grain*	*Experiment*	*Temperature (°C)*	*Agitation (rpm)*	*Amount*	*Lactic Acid (LA) g/L*
500	WKGCOL3	3	37	137	Maximum	11,34
		8	20	80	Minimun	1,24
	WKGCH2	3	37	137	Maximum	11,00
		8	20	80	Minimun	1,60
250	WKGCOL3	3	37	137	Maximum	9,30
		8	20	80	Minimun	1,30
	WKGCH2	3	37	137	Maximum	11,00
		8	20	80	Minimun	1,40

Grain	*Experiment*	*Temperature (°C)*	*Agitation (rpm)*	*Amount*	*Lactic Acid Bacteria - LAB (Abundance)*
WKGCOL3	7	23	23	Maximum	69067,00
	3	37	137	Minimun	39702,00
WKGCH2	1	23	137	Maximum	62942,00
	3	37	137	Minimun	33355,00

Volume (mL)	*Grain*	*Experiment*	*Temperature (°C)*	*Agitation (rpm)*	*Amount*	*Ethanol (EtOH) g/L*
500	WKGCOL3	3	37	137	Maximum	25,50
		8	20	80	Minimun	7,00
	WKGCH2	3	37	137	Maximum	7,00
		8	20	80	Minimun	21,00
250	WKGCOL3	2	30	160	Maximum	20,42
		8	20	80	Minimun	9,21
	WKGCH2	2	30	160	Maximum	19,57
		8	20	80	Minimun	7,62

Grain	*Experiment*	*Temperature (°C)*	*Agitation (rpm)*	*Amount*	*Yeast -LEV (Abundance)*
WKGCOL3	1	23	137	Maximum	55426,00
	2	30	160	Minimun	10866,00
WKGCH2	1	23	137	Maximum	39161,00
	2	30	160	Minimun	15002,00

Volume (mL)	*Grain*	*Experiment*	*Temperature (°C)*	*Agitation (rpm)*	*Amount*	*Acetic Acetic (AA) g/L*
500	WKGCOL3	3	37	137	Maximum	0,95
		8	20	80	Minimun	0,40
	WKGCH2	3	37	137	Maximum	0,70
		8	20	80	Minimun	0,15
250	WKGCOL3	2	30	160	Maximum	1,01
		8	20	80	Minimun	0,26
	WKGCH2	2	30	160	Maximum	0,62
		8	20	80	Minimun	0,22

Grain	*Experiment*	*Temperature (°C)*	*Agitation (rpm)*	*Amount*	*Acetic Acetic (AA) g/L*
WKGCOL3	4	40	80	Maximum	303,00
	5	37	23	Minimun	44,00
WKGCH2	4	40	80	Maximum	484,00
	2	30	160	Minimun	59,00

Table G. *Summary table shows the data for the maximum and minimum amounts of sugars (GLUC, FRUC, FRUC), for the analysis of the trends of sugar consumption between the experiments (250 and 500 mL) and of the total sugar content in the final fermented beverage.*

Volume (mL)	*Grain*	*Experiment*	*Temperature (°C)*	*Agitation (rpm)*	*Amount*	*Glucose (GLUC) g/L*
500	**WKGCOL3**	8	20	80	Maximum	11,65
		3	37	137	Minimum	2,15
	WKGCH2	8	20	80	Maximum	12,80
		3	37	137	Minimum	4,60
250	**WKGCOL3**	8	20	80	Maximum	10,70
		2	30	160	Minimum	3,83
	WKGCH2	8	20	80	Maximum	11,97
		2	30	160	Minimum	4,97
Volume (mL)	***Grain***	***Experiment***	***Temperature (°C)***	***Agitation (rpm)***	***Amount***	***Fructose (FRUC) g/L***
500	**WKGCOL3**	4	40	80	Maximum	11,45
		7	23	23	Minimum	3,90
	WKGCH2	4	40	80	Maximum	16,50
		2	30	160	Minimum	8,75
250	**WKGCOL3**	4	40	80	Maximum	19,67
		2	30	160	Minimum	4,99
	WKGCH2	4	40	80	Maximum	23,40
		2	30	160	Minimum	6,43
Volume (mL)	***Grain***	***Experiment***	***Temperature (°C)***	***Agitation (rpm)***	***Amount***	***Sucrose (SUCR) g/L***
500	**WKGCOL3**	8	20	80	Maximum	61,90
		3	37	137	Minimum	6,35
	WKGCH2	8	20	80	Maximum	48,55
		3	37	137	Minimum	11,35
250	**WKGCOL3**	8	20	80	Maximum	35,72
		4	40	80	Minimum	8,16
	WKGCH2	8	20	80	Maximum	38,95
		3	37	137	Minimum	7,97

8.6. APPENDIX: Definitive metabarcoding analysis for identification and optimization

Table A. *Relative abundance (%) according to the metagenomic analysis performed for the dynamic study, given for each bacterial genus and species in (WKGCOL3).*

		WKGCOL3 microorganism species											
Experiment	**Time (h)**	*Bacillus nealsonii*	*Lactobacillus satsumensis*	*Oenococcus alcoholitoler*	*Lactobacillus perolens*	*Oenococcus oeni*	*Lactobacillus diolivorans*	*Oenococcus kitaharae*	*Lactobacillus parabuchneri*	*Lactobacillus hilgardii*	*Lactobacillus casei/paraca*	*Lactobacillus harbinensis*	*Lactobacillus ghanensis*
EXP1	**48h**	0.00	0.00	3.52	1.23	2.20	4.26	9.45	8.27	10.46	9.82	11.08	38.65
	96h	0.00	0.00	2.32	1.15	2.82	4.78	7.98	9.49	10.50	11.24	11.99	36.37
EXP2	**48h**	0.00	0.00	3.46	1.12	4.44	8.17	2.54	7.86	17.93	9.65	16.28	27.44
	96h	0.00	0.00	0.68	1.91	1.48	2.18	0.70	3.38	9.86	7.81	8.79	62.37
EXP3	**48h**	0.00	0.00	0.77	2.21	4.13	7.35	0.76	8.52	14.61	16.23	16.83	28.12
	96h	0.00	0.00	0.35	0.82	15.36	7.96	0.16	13.95	7.37	10.11	17.13	26.52
EXP4	**48h**	0.00	0.00	0.47	1.19	2.29	7.55	1.01	9.65	12.76	20.51	16.25	27.41
	96h	0.00	0.00	0.24	0.41	2.21	2.82	0.19	9.80	9.15	18.65	27.75	28.55
EXP5	**48h**	0.00	0.00	1.47	1.20	4.36	5.67	0.94	12.05	14.07	12.57	28.57	18.77
	96h	0.00	0.01	0.28	2.09	8.92	11.41	0.25	12.06	21.44	9.90	20.89	12.49
EXP6	**48h**	0.00	0.00	2.26	0.51	3.30	5.52	1.20	10.58	14.42	12.58	16.84	31.98
	96h	0.00	0.00	0.87	0.85	5.19	4.16	0.60	11.14	15.06	14.09	21.21	26.24
EXP7	**48h**	0.00	0.00	3.76	1.01	2.50	7.70	7.10	7.63	11.18	11.79	11.62	34.62
	96h	0.01	0.41	0.02	1.21	0.18	3.13	5.54	5.13	7.83	7.93	5.76	62.26
EXP8	**48h**	0.00	0.00	3.90	1.49	3.06	6.99	4.37	8.79	11.81	15.57	16.13	26.99
	96h	0.00	0.00	2.47	1.93	3.82	5.13	4.18	9.28	11.08	12.15	15.38	32.49
EXP 9-13	**48h**	0.00	0.00	1.92	1.09	2.90	7.68	1.39	7.02	11.20	11.81	17.27	36.68
	96h	0.00	0.00	1.24	0.98	3.55	4.59	1.36	7.31	15.06	12.09	17.40	31.00
Average Relative Abundance (%)		0.00	0.02	1.67	1.25	4.04	5.95	2.76	8.99	12.54	12.47	16.51	32.72

	WKGOL3 microorganism genus			
Average Relative Abundance (%)	*Gluconobacter*	*Acetobacter*	*Oenococcus*	*Lactobacillus*
	0.35	0.32	8.35	90.8

Table B: *Relative abundance (%) according to the metagenomic analysis performed for the dynamic study, given for each bacterial genus and species in (WKGCH2).*

		WKGCH2 microorganism species										
Experiment	**Time (h)**	*Bacillus nealsonii*	*Lactobacillus satsumensis*	*Oenococcus alcoholitoler*	*Lactobacillus perolens*	*Oenococcus oeni*	*Lactobacillus diolivorans*	*Oenococcus kitaharae*	*Lactobacillus parabuchneri*	*Lactobacillus hilgardii*	*Lactobacillus casei/paraca*	*Lactobacillus harbinensis*	*Lactobacillus ghanensis*
EXP1	**48h**	0.00	0.15	0.01	1.98	0.06	2.24	8.65	4.80	5.43	13.14	6.68	55.40
	96h	0.02	0.32	0.07	1.19	0.12	2.32	6.82	6.93	6.72	11.34	7.19	54.39
EXP2	**48h**	0.03	0.13	0.07	1.55	0.17	4.16	7.62	8.07	7.53	20.93	12.22	35.82
	96h	0.02	0.04	0.01	2.37	0.28	7.82	2.74	12.46	6.78	19.28	13.40	33.14
EXP3	**48h**	0.01	0.08	0.08	3.32	0.38	5.77	1.69	9.68	9.42	23.12	18.28	25.96
	96h	0.01	0.04	0.02	1.80	0.78	8.61	0.25	13.42	5.57	11.68	13.38	43.92
EXP4	**48h**	0.00	0.27	0.04	3.30	0.19	3.91	0.29	9.49	6.39	13.34	9.98	52.21
	96h	0.01	0.11	0.00	0.42	0.21	4.06	0.25	13.55	5.90	18.00	16.56	40.69
EXP5	**48h**	0.07	0.16	0.11	3.51	1.15	5.26	2.78	9.28	9.08	15.95	17.54	34.58
	96h	0.00	0.07	0.03	1.92	0.27	6.01	1.51	7.01	8.66	12.99	9.02	51.26
EXP6	**48h**	0.17	0.14	0.23	2.25	0.44	4.73	4.70	8.91	9.03	16.23	14.20	38.12
	96h	0.05	0.03	0.06	4.09	0.26	4.21	2.78	6.76	8.34	13.63	10.67	47.93
EXP7	**48h**	0.01	0.09	0.02	1.74	0.13	2.38	8.60	5.54	4.42	10.58	6.69	58.76
	96h	0.00	0.00	2.71	1.24	2.76	6.89	5.06	8.05	10.29	8.61	15.17	38.19
EXP8	**48h**	0.01	0.15	0.05	3.56	0.14	3.47	8.62	8.13	5.54	14.38	10.28	43.06
	96h	0.03	0.08	0.03	1.83	0.20	4.01	11.88	7.02	6.52	12.13	9.41	44.20
EXP 9-13	**48h**	0.02	0.11	0.02	1.67	0.21	3.09	3.99	6.13	6.68	21.16	8.70	45.56
	96h	0.02	0.09	0.02	1.75	0.20	3.55	7.93	6.57	6.60	16.65	9.05	44.88
Average Relative Abundance (%)		0.03	0.11	0.20	2.19	0.44	4.58	4.79	8.43	7.16	15.17	11.58	43.78

	WKGOL3 microorganism genus			
Average Relative Abundance (%)	*Gluconobacter*	*Acetobacter*	*Oenococcus*	*Lactobacillus*
	0.53	0.41	5.02	93.33

Table C. *Relative abundance (%) according to the metagenomic analysis performed for the dynamic study, given for each Fungal genus and for each species in WKGCOL3.*

			WKGCOL3 microorganism species				
	Experiment	**Time (h)**	***Saccharomyces cerevisiae***	***Dekkera bruxellensis***	***Zygotorulaspora florentina***	***Saccharomycodes ludwigii***	***Schizosaccharomyces pombe***
Relative Abundance (%)	**EXP1**	**48h**	73.23	22.49	2.44	3.35	0.26
		96h	20.41	75.25	3.03	2.07	0.14
	EXP2	**48h**	90.86	3.67	0.01	1.89	2.01
		96h	90.21	5.06	0.00	1.79	1.56
	EXP3	**48h**	91.03	4.07	0.01	3.05	1.48
		96h	92.39	3.45	0.00	3.01	2.38
	EXP4	**48h**	88.75	5.82	0.00	1.50	4.02
		96h	37.18	62.53	0.01	2.32	0.00
	EXP5	**48h**	91.59	4.14	0.02	3.28	1.34
		96h	91.32	4.29	0.00	2.81	1.93
	EXP6	**48h**	91.01	4.90	0.03	2.10	1.89
		96h	93.00	2.76	0.00	2.17	1.18
	EXP7	**48h**	57.92	38.04	1.82	1.73	0.28
		96h	58.94	23.01	15.85	2.46	0.48
	EXP8	**48h**	88.02	5.50	0.71	1.58	1.60
		96h	54.86	40.12	2.82	2.10	0.26
	EXP 9-13	**48h**	88.82	5.82	0.09	2.74	2.37
		96h	88.11	4.55	0.05	3.01	3.49

WKGCOL3 microorganism genus					
Average Relative Abundance (%)	***Saccharomyces***	***Dekkera***	***Zygotorulaspora***	***Saccharomycodes***	***Schizosaccharomyces***
	77.09	17.77	1.49	1.48	2.07

Table D. *Relative abundance (%) according to the metagenomic analysis performed for the dynamic study, given for each Fungal genus and for each species in WKGCH2.*

			WKGCH2 microorganism species				
	Experiment	**Time (h)**	***Saccharomyces cerevisiae***	***Dekkera bruxellensis***	***Zygotorulaspora florentina***	***Saccharomycodes ludwigii***	***Schizosaccharomyces pombe***
Relative Abundance (%)	**EXP1**	**48h**	65.99	16.98	2.44	0.94	0.09
		96h	46.32	14.68	3.44	0.59	0.17
	EXP2	**48h**	92.34	2.70	4.44	1.89	2.48
		96h	95.33	1.26	5.44	1.50	1.51
	EXP3	**48h**	91.05	3.37	6.44	2.10	3.33
		96h	91.38	4.43	7.44	1.58	2.52
	EXP4	**48h**	32.46	66.46	8.44	0.35	0.13
		96h	83.50	15.87	9.44	0.06	0.17
	EXP5	**48h**	91.86	3.96	10.44	2.17	1.68
		96h	89.01	5.95	11.44	2.10	2.81
	EXP6	**48h**	91.31	3.95	12.44	1.79	2.18
		96h	92.04	3.47	13.44	2.32	1.50
	EXP7	**48h**	29.17	57.57	14.44	0.34	0.11
		96h	67.29	28.72	15.44	0.82	0.22
	EXP8	**48h**	36.81	40.64	16.44	0.73	0.17
		96h	61.32	24.32	17.44	1.01	0.16
	EXP 9-13	**48h**	88.70	4.62	18.44	2.56	2.82
		96h	89.67	3.46	19.94	2.66	3.14

WKGCH2 microorganism genus					
Average Relative Abundance (%)	***Saccharomyces***	***Dekkera***	***Zygotorulaspora***	***Saccharomycodes***	***Schizosaccharomyces***
	74.20	16.85	5.86	1.33	1.42

***Table E**. The ANOVA table shows the test statistics for the response variables (Lactobacillus, Acetic Acid Bacteria and Yeasts) with linear and quadratic fits, respectively, for WKGCH2 and WKGCOL3 in the 250 mL experiments.*

Source	***Df***	***Lactic Acid Bacteria (LAB)***	***Acetic Acid Bacteria (AAB)***	***Yeast***
		p-value	***p-value***	***p-value***
Model WKGCH2	5	0.0350	0.0010	0.0019
X1-Temperature (°C)	1	0.0093	0.0170	0.0041
X2-Agitation (RPM)	1	0.3241	0.6583	0.2330
X1X2	1	0.4616	0.0439	0.0694
X1²	1	0.0292	0.0001	0.0004
X2²	1	0.2787	0.1319	0.1470
Statistical parameters				
R²		0,7263	0,8934	0,8754
R² adj		0,5552	0,8267	0,7976
Pred-R²		-0,909	0,299	0,1148
Adeq Precision		6,4312	12,2551	9,8029
CV. %		15,488	28,2917	15,92

Source	***Df***	***Lactic Acid Bacteria (LAB)***	***Acetic Acid Bacteria (AAB)***	***Yeast***
		p-value	***p-value***	***p-value***
Model WKGCOL3	5	0.0659	0.5467	0.5905
X1-Temperature (°C)	1	0.0254	0.6582	0.595
X2-Agitation (RPM)	1	0.9238	0.9588	0.5761
X1X2	1	0.1137	0.2021	0.3357
X1²	1		0.2054	0.7622
X2²	1		0.7059	0.179
Statistical parameters				
R²		0,498	0.8269	0,3493
R² adj		0,347	0.7188	-0,0574
Pred-R²		-0,741	-0.2243	-3,5306
Adeq Precision		6,715	83.345	2,6838
CV. %		13,236	25.63	46,9624

Table F. *The standard deviations (SD) are shown for each type of bacteria and yeast under each of the experimental conditions (experiments 1, 2, 3, 4, 5, 7, 8, and 13) according to the CCD, calculated over the different evaluated times. (48 y 96 hours). Values greater than 1 indicate a greater dispersion or variability between the two times of the experiment.*

Genera (SD) WKGCH2 48-96 h											
Genus	**EXP1**	**EXP2**	**EXP3**	**EXP4**	**EXP5**	**EXP6**	**EXP7**	**EXP8**	**EXP13**	**General SD 48h**	**General SD 96 h**
Lactobacillus	0,236	3,606	2,058	0,027	1,333	2,461	1,163	2,595	1,247	3,250	4,676
Oenococcus	1,212	3,416	0,778	0,045	1,581	2,588	1,262	2,332	0,933	3,128	4,221
Gluconobacter	0,113	0,252	1,097	0,025	0,290	0,031	0,059	0,026	0,371	0,647	0,457
Acetobacter	0,851	0,069	0,179	0,011	0,002	0,061	0,033	0,223	0,054	0,310	0,536
Genera (SD) WKGCOL3 48-96 h											
	EXP1	**EXP2**	**EXP3**	**EXP4**	**EXP5**	**EXP6**	**EXP7**	**EXP8**	**EXP13**	**General SD 48h**	**General SD 96 h**
Lactobacillus	1,054	5,341	7,203	0,804	1,877	0,045	5,212	0,248	2,916	3,893	4,589
Oenococcus	1,449	5,358	7,211	0,800	1,889	0,074	5,396	0,612	0,053	3,881	4,495
Gluconobacter	0,008	0,008	0,002	0,003	0,000	0,007	0,137	0,004	2,067	0,009	0,968
Acetobacter	0,387	0,009	0,006	0,001	0,012	0,022	0,041	0,865	0,902	0,086	0,587
Yeast (SD) WKGCH2 48-96 h											
Genus	**EXP1**	**EXP2**	**EXP3**	**EXP4**	**EXP5**	**EXP6**	**EXP7**	**EXP8**	**EXP13**	**General SD 48h**	**General SD 96 h**
Saccharomyces	13,909	2,114	0,233	36,091	2,015	0,516	26,955	17,331	0,686	28,298	17,100
Dekkera	1,626	1,018	0,750	35,773	1,407	0,339	20,400	11,540	0,820	25,707	10,029
Zygotorulaspora	0,707	0,707	0,707	0,707	0,707	0,707	0,707	0,707	1,061	5,477	5,570
Saccharomycodes	0,247	0,276	0,368	0,205	0,049	0,375	0,339	0,198	0,071	0,844	0,859
Schizosaccharomyces	0,057	0,686	0,573	0,028	0,799	0,481	0,078	0,007	0,226	1,327	1,236
Yeast (SD) WKGCOL3 48-96 h											
Genus	**EXP1**	**EXP2**	**EXP3**	**EXP4**	**EXP5**	**EXP6**	**EXP7**	**EXP8**	**EXP13**	**General SD 48h**	**General SD 96 h**
Saccharomyces	37,349	0,460	0,962	36,465	0,191	1,407	0,721	23,448	0,502	11,502	27,631
Dekkera	37,307	0,983	0,438	40,100	0,106	1,513	10,628	24,480	0,898	11,889	28,214
Zygotorulaspora	0,417	0,007	0,007	0,007	0,014	0,021	9,921	1,492	0,028	0,926	5,193
Saccharomycodes	0,905	0,071	0,028	0,580	0,332	0,049	0,516	0,368	0,191	0,748	0,439
Schizosaccharomyces	0,085	0,318	0,636	2,843	0,417	0,502	0,141	0,948	0,792	1,131	1,184

8.7. APPENDIX: Comparative Analysis of Biomass Growth

Table A. *This table describes the maximum and minimum amounts obtained in the central composite design (CCD) optimization experiments, showing the percentage of biomass yield of the different WKGs evaluated and selected (WKGCOL3, WKGCH2, WKGALE) according to the amount of initial inoculum (80g/L).*

Volume (mL)	*Grain*	*Experiment*	*Temperature (°C)*	*Agitation (rpm)*	*Amount*	*Biomass g/L*	*Yield %*
500	**WKGCOL3**	7	23	23	Maximum	150,32	87,90
		4	40	80	Minimum	97,78	22,23
	WKGCH2	7	23	23	Maximum	123,21	54,01
		3	37	137	Minimum	91,47	14,34
250	**WKGCOL3**	2	30	160	Maximum	123,48	54,35
		5	37	23	Minimum	99,97	24,96
	WKGCH2	1	23	137	Maximum	121,17	51,46
		3	37	23	Minimum	92,81	16,01