BIOANCIENT ™: FERMENTATION AND ITS RELATION TO PROTEIN, BIOACTIVE PEPTIDES AND VITAMIN B-COMPLEX

A Project Paper

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Professional Studies in Agriculture and Life Sciences

Field of Food Science

By Sheren Leonita Winarto

May 2019

ABSTRACT

The rise of chronic diet-related diseases due to poor lifestyle behaviors have led to consumers being much more health-conscious with regards to the nutrients that they are consuming. More frequently now than ever, consumers are turning to the nutrient labels as a guide to achieve daily nutrient intake goals and be informed of the ingredients used in products. They are also seeking healthier alternatives to the products that they frequently consume, driving the demand for clean labelled products. In addition, consumers are also seeking nutritionally dense foods, commonly called 'superfoods', to nourish their well-being, and are eager in educating themselves in relation to the components of the foods that make them highly nutritious. These have led to trends in consuming foods that are rich in antioxidants, collagen, vitamins or anti-aging ingredients. Vitamin B-complex are one of the most sought-after ingredients in a product. One top of that, recently, bioactive peptides have been identified to have multiple promising health beneficial activities making them desirable components of a beverage. Hence, the study herein takes advantage of scientific and technological knowledge to give rise to a beverage that is both rich in nutrients and delicious, named BioAncient. BioAncient is a fermented rice beverage that contains proteins, bioactive peptides and vitamin B-complex from the interactions between yeast, mold (*A. oryzae*), and rice during fermentation. The results show that the presence of mold (*A. oryzae*) was important in significantly increasing concentrations of bioactive peptides LSP, YW, and vitamin B6, whilst the presence of yeast was important in significantly increasing concentrations of protein, bioactive peptide FR, vitamin B1, vitamin B3, and vitamin B6. Mold (*A. oryzae*) and yeast were shown to have a symbiotic relationship, especially in the synthesis of vitamin B6. Fermenting the beverage at 22°C worked best at achieving the highest concentration of bioactive peptide FR. On the other hand, fermenting the beverage at 30°C produced the highest concentrations of protein and vitamin

B6. In addition, fermenting the beverage at 33°C yielded the highest concentration of vitamin B1. Other nutrients, such as bioactive peptide VY and vitamin B3, also showed increased concentrations over 28°C-33°C. However, fermentation temperatures 22°C-33°C does not seem to have significant effect on the concentrations of bioactive peptides LSP and YW, and vitamin B5. Fermenting the beverage for 2 days worked best at achieving the highest concentration of bioactive peptide VY. Other nutrients, such as bioactive peptides LSP, FR, and YW, vitamin B1 and vitamin B6, saw its increased concentrations over 5-7 days. However, fermentation durations of 2-7 days do not seem to have significant effect on the concentrations of protein, vitamin B3, and vitamin B5. The intent of this study will be a step closer in understanding the relationship between mold (*A. oryzae*) and yeast, and use it to our advantage to enhance nutrients in our foods naturally.

BIOGRAPHICAL SKETCH

Sheren Leonita Winarto is from Surabaya, East Java, Indonesia. She received a Bachelor of Science in Food Science and Technology from University of California, Davis in 2017.

She is currently completing her Master of Professional Studies degree in Food Science, emphasizing on Food Chemistry and Product Development at Cornell University under the supervision of Dr. Alireza Abbaspourrad. Her time in Cornell University has been spent focusing on quantifying bioactive peptides and vitamin B-complex, as well as exploring the factors which influences these measurements to optimize nutrient content in her fermented rice beverage. Apart from her research and commitments in her classes, she is also actively involved in the Dyson School of Applied Economics and Management, co-leading the Indonesia Cau Chocolate Team under the Student Multidisciplinary Applied Research Teams (SMART) Program. She is also a part of the Smith Family Business Initiative and the Women Entrepreneurs (W.E) Cornell Program. She was also a participant of the 2019 Digital Agriculture Hackathon, where her team, Insect Insight, won the Grand Prize formulating an idea and technology that involved the use of Black Soldier flies as an alternative to commercial fish feed. Her future plans include a career in product development and bringing innovation and advancement to the food industry.

I would like to thank my parents, Winarto and Melissa, for their unwavering support and inspirations. I wouldn't be able to achieve and be the person I am today without you. Thank you for everything that you have done for me.

ACKNOWLEDGEMENTS

I would like to express my greatest gratitude to my advisor Dr. Alireza Abbaspourrad, who is a great mentor and scientist. His unwavering support and advice have driven and molded me to become a better scientist and person.

I would also like to express my tremendous appreciation to Zhong Zhang. Your warm advice and encouragements, and dedication was a huge drive in moving this project forward. Your faith and confidence in me are something I will carry for the rest of my life.

Also, I want to sincerely thank members of the Abbaspourrad lab for helping through this project, in particular Peter Lawrence and Mojtaba Enayati.

Lastly, I would like to express my thanks to the Food Science department and staff for the support given to me throughout my time in Cornell.

Thank you all for a life-changing and unforgettable experience here in Cornell.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Background

Over the past years, the rise of chronic diet-related diseases has led to consumers being much more health-conscious with regards to the nutrition that they are consuming. Vitamin B-complex, one of the most essential water-soluble vitamins, is one of the most sought-after ingredients in a product. On the other hand, bioactive peptides have shown to have multiple health benefits, including the modulation of the body's physiological functions, making it a very desirable component in beverages (Chakrabarti, Guha, & Majumder, 2018). As consumers are pushing for transparency and naturality of ingredients used in food products, many are turning towards nature for solutions. Hence, BioAncient utilizes mold (*A. oryzae*) and yeast, nature's very own hydrolyzers, to produce bioactive peptides and vitamin B-complex from rice grains, through the process of fermentation.

Vitamin B-complex is one of the most essential water-soluble vitamins consisting of vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B3 (niacin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin B7 (biotin), vitamin B9 (folic acid) and vitamin B12 (cobalamins). Each of these vitamins have their own unique structures and performs different functions important to the human body. As these vitamins are essential, they must be replenished daily as they are not synthesized by the body or stored in fat reserves. Their functions include maintenance of myelin, healthy red blood cells and homocysteine levels, boost immune function, hormone production and

regulate cell growth ("Vitamin B Complex.", 2005). In addition to their health benefits, vitamin B-complex are commonly added to food products as additives to replace amounts lost due to processing or exposure to light and oxygen, or to maintain freshness and improve food safety.

Bioactive peptides are fragmented proteins that imposes beneficial impact on the bodily functions and conditions, and may ultimately improve health conditions (Kitts & Weiler, 2003). These peptides are inactive when imbedded within the parent protein, but when released through the hydrolysis by digestive enzymes, or the action of proteolytic enzymes from microorganisms or plants, it exhibits specific properties depending on their amino acid sequences (**Table 1**) that may benefit body systems such as cardiovascular, digestive, immune and nervous systems (Korhonen & Pihlanto, 2006). Due to their potential role in boosting human health, bioactive peptides have gathered many interests over the past few years, notably in milk proteins and fermented dairy products. Hence, it is of great interest to investigate bioactive peptides in other fermented non-dairy alternatives, such as BioAncient, a fermented beverage made out of rice.

Table 1. Functions of Bioactive Peptides. (Han & Xu, 2011)

Sequence of Bioactive Peptides	Description of Function
VY, TVY, GF, LSP, WL, LPP, IPP, YG, YW, TVY, VYPFPG, VYP, LTF, TF, LVR, LQQ, KW, VPP, YL, LF, ILP, LLP, LPQ, NPP, KP, APL, LRP, VPP, LKP, LVQ	Angiotensin-converting enzyme (ACE)
FR, AH, HL, LHV, LLHH, LHQ, LDR, LLPH	Antioxidative
LP,	Dipeptidyl-aminopeptidase IV inhibitor
VF, GPR	Dipeptidyl-aminopeptidase IV inhibitor; ACE
LP, FR	Dipeptidyl-aminopeptidase IV inhibitor; Antioxidative

WL, LLHH, HLL	ACE; Antioxidative
LNP	Antihypertensive
FPP	Bitter
FP	Bitter, ACE
YPR, LPYPR	Hypocholesterolemic
YGG	Immunomodulatory
EEE	Umami; anti-bitter

Fermentation

Fermentation is the process whereby bacteria, yeasts or other microorganisms break down sugars into alcohol and carbon dioxide, and in some cases secondary metabolites with health benefits are produced, including bioactive peptides and vitamin B-complex (Stanton, Ross, Fitzgerald, & Sinderen, 2005). *Aspergillus oryzae* can produce exoproteases and endoproteases, which can cleave bonds and generate peptides with functional properties (Zanutto-Elgui, et al., 2019). Also, yeast is able to secrete proteases, which can assist in the release of proteins from the rice grains (Ogrydziak, 1993).

Research Intent

The present study attempts to quantify protein, vitamin B-complex and bioactive peptides imparted by mold (*A. oryzae*) and yeast activities on rice grains and enrich these beneficial elements in a nutritious beverage. In addition, the parameters that affects fermentation, such as time and temperature, are also explored in order to further understand the relationship between mold (*A. oryzae*), yeast and the yield of protein, bioactive peptides and vitamin B-complex. Ultimately, the goal is to create a nutritious and delicious beverage packed with protein, vitamin B-complex and

bioactive peptides for those who are deficient in vitamin B-complex due to various absorption issues and dietary choices, and also to nourish and improve overall health.

CHAPTER 2

MATERIALS & METHODS

Ingredients of BioAncient & Analytical Standards

Rice grains used in this study was Sho-Chiku-Bai Sweet Rice from Koda Farms (San Joaquin Valley, CA). *A. oryzae* used was purchased as Sake Homebrew Starter from Vision Brewing (Nedlands, Western Australia). Yeast used was acquired as Shanghai Yeast Balls from Golden Lion (USA). Stable Lowry Protein Assay kit was purchased from Bio Basic (Amherst, NY, USA). All vitamin B-complex (B1, B3, B5, B6) and peptide standards were of analytical standard grade and purchased from Millipore Sigma (St. Louis, MO, USA). All ingredients used were food grade and all other chemicals utilized were reagent grade. Beverage was made in a certified food grade lab.

Making of BioAncient

The beverage was prepared in a food-grade laboratory. The preparation process was as follows: 150g rice was rinsed three times and then soaked in water for 4 h, and drained. The soaked rice was then steamed in a steam convection oven (Cuisinart, China) for 30 min at 210 °F. After cooling to 30 °C, 200 g steamed rice was inoculated with a cultured liquid consisting of 0.5 g *A. oryzae*, 0.5 g yeast, and 200 g water. Incubation was carried out at 22 °C - 33 °C for 2 - 7 days in a water bath facilitated with a sous vide machine (Wancle SVC001 Sous Vide Cooker, China). After incubation, the mixture was stirred well and placed in a 55°C water bath (Fisher Scientific, U.S.A)

for 1 h to extract the proteins. The mixture was then strained through a cheesecloth and pasteurized by placing in a water bath at 90°C for 10 min.

Quantification of Protein Using Lowry's Protein Assay

Protein concentrations were quantified with reference to standards of bovine serum albumin (BSA). The standards were prepared according to the manufacturer's manual with concentrations from 0.00 mg/mL - 0.80 mg/mL. Samples were centrifuged using (Eppendorf MiniSpin, Germany) at 12.0 rpm for 10 mins, obtaining the supernatant. Samples were diluted 100x and prepared according to the manufacturer's manual. Subsequently, 20 µL of sample were mixed with 100 µL of Stable Copper Reagent and left to incubate at room temperature (23 °C - 25 °C) for 10 mins. It is then mixed with 10 µL of Folin-Phenol Reagent (1N) and left to incubate at room temperature (24 °C) for 30 mins. The above prepared standards and samples were prepared into triplicate wells of a 96-well polystyrene microplate. The visible light absorption spectra of the standards and samples were obtained at 750 nm using SpectraMax iD3 Multi-Mode microplate reader from Molecular Devices (San Jose, CA). The average 750nm absorbance value of the blank standard replicates were subtracted from the 750nm values of the BSA standards and samples. A standard curve was prepared and used to determine the protein concentration of each unknown sample.

Quantification of Vitamin B-Complex (B1, B3, B5, B6) & Bioactive Peptides using HPLC/(+) ESI-SRM

The bioactive peptides and vitamin B-complex (B1, B3, B5, B6) are quantified with reference to standards using HPLC/(+) ESI-SRM. Samples were centrifuged using (Eppendorf MiniSpin, Germany) at 12.0 rpm for 10 mins to obtain the supernatant, and then diluted twice in DI water.

Bioactive peptide standards were prepared using HPLC peptide standard mixture, diluted to 0.00010 mg/mL – 0.1 mg/mL with Milli-Q filtered water. Vitamin B-complex standards (B1, B3, B5, B6) were prepared by diluting to 0.0001 mg/mL – 0.01 mg/mL with Milli-Q filtered water.

Samples were run by Aglient 1100 HPLC coupled to a Thermo LTQ MSn ion trap with an ESI ion source. The isocratic mobile phase consisted of 0.01% formic acid at a flow rate of 0.6 ml per minute. The HPLC column was a Waters Cortecs C18 2.7µm 4.6x100mm. The column was held at room temperature. The injection volume was 10µL for all samples. The ion source parameters for positive ion mode were as follows: Sheath gas flow rate 35, Aux gas flow rate 15, spray voltage 4.5, capillary temperature 325°C, capillary voltage 35.0 V, and tube lens 110 V. Total run time for all samples and scan types was 20 minutes. Samples were run using SRM scan mode. Please refer to **Table 2** for mass transitions monitored.

Table 2. SRM mass transitions for Vitamins and Peptides measured.

Vitamin	Parent mass	Transition mass
B1	266.3	122.3
B2	377.3	243.3
B3	123.3	123.3
B5	220.3	90.3
B6	168.3	168.3
B9	442.3	295.3
Peptides		
YW	367.3	186.3
VY	280.3	136.3
LSP	315.3	227.3
FR	321.3	247.3
LLPH	262.3	235.3
YPR	434.3	175.3

A standard curve was prepared and used to determine the protein concentration of each unknown sample.

Statistical Analysis

The statistical analysis was performed with Minitab Express (Minitab, Inc., State College, Pennsylvania, USA). Means, standard deviations and significant differences of results were obtained by variance analysis (ANOVA). Significance level of 0.05 was used in this study.

CHAPTER 3

DISCUSSION

Three different variables were explored to understand the relationship between mold (*A. oryzae*), yeast and the levels of protein, vitamin B-complex and bioactive peptides: Type of starter culture, fermentation temperature and fermentation duration.

Quantification of Protein

Different types of starter cultures were used in the beverages and quantified for proteins using the Lowry's protein assay: control (no organisms), mold (*A. oryzae*), yeast, and mold (*A. oryzae*) and yeast. The protein content of the beverage with mold and yeast was the highest compared to the rest, mold and yeast (2.33 g/100 ml) > yeast (2.16 g/100 ml) > control (0.54 g/100 ml) > mold (0.13 g/100 mL) (**Fig. 1**). There was a significant difference between the protein contents of the beverages with both mold and yeast and yeast only, and the beverages with mold and the control. This shows that yeast may directly affect the beverage's protein content. Following this observation, parameters of fermentation were explored to further study the relationship between mold (*A. oryzae*), yeast and the concentrations of protein.

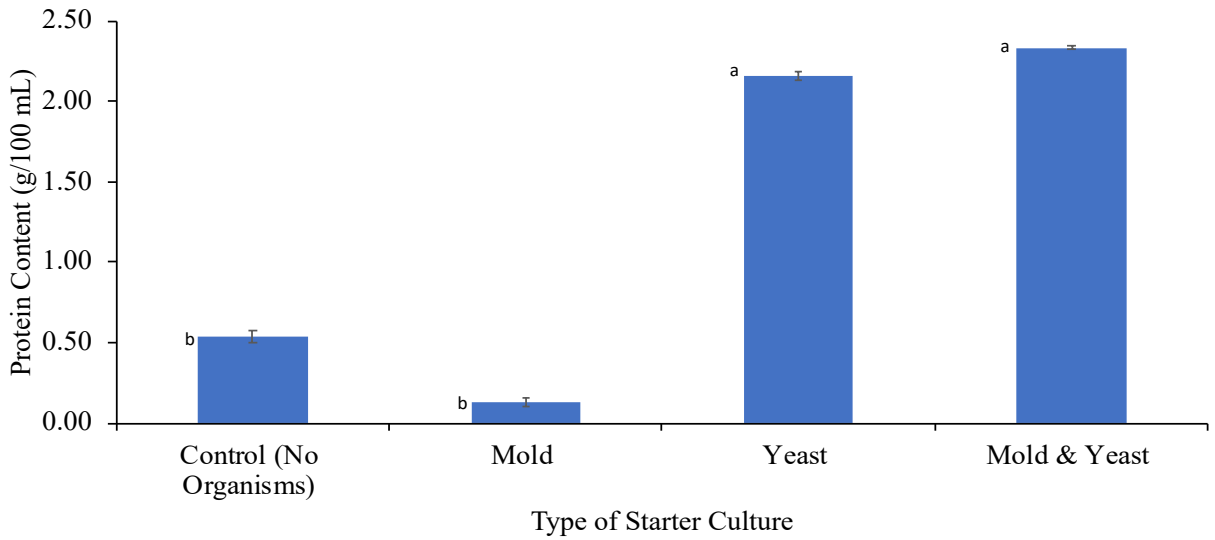


Figure 1. Protein content of beverages made with different starter cultures (control, mold (*A. oryzae)*, yeast, mold (*A. oryzae*) and yeast) fermented at 28°C for a duration of 5 days, using Lowry's Protein Assay.

The rice beverages were fermented at different temperatures, ranging from 22°C - 33°C, with mold and yeast to maximize the protein content produced, as observed from above (**Fig. 1**). The protein content of the beverage fermented at 30 °C was the highest compared to the rest: 30°C (2.87 g/100 mL) > 28°C & 33°C (2.33 g/100 mL) > 25°C (1.98 g/100 mL) > 22°C (1.66 g/100 mL) (**Fig. 2**). There was a significant difference between the protein content of the beverage fermented at 30°C and the others. There was also a significant difference between the protein content of the beverage fermented at 22°C and the protein content of the beverages fermented at 28°C and 33°C. However, there was no significant difference between the protein contents of the beverages fermented at 25°C, 28°C, and 33°C. Also, there was no significant difference between the protein contents of the beverages fermented at 22°C and 25°C. Following this observation, durations of fermentation were explored to further study the relationship between mold (*A. oryzae*), yeast and the concentrations of protein.

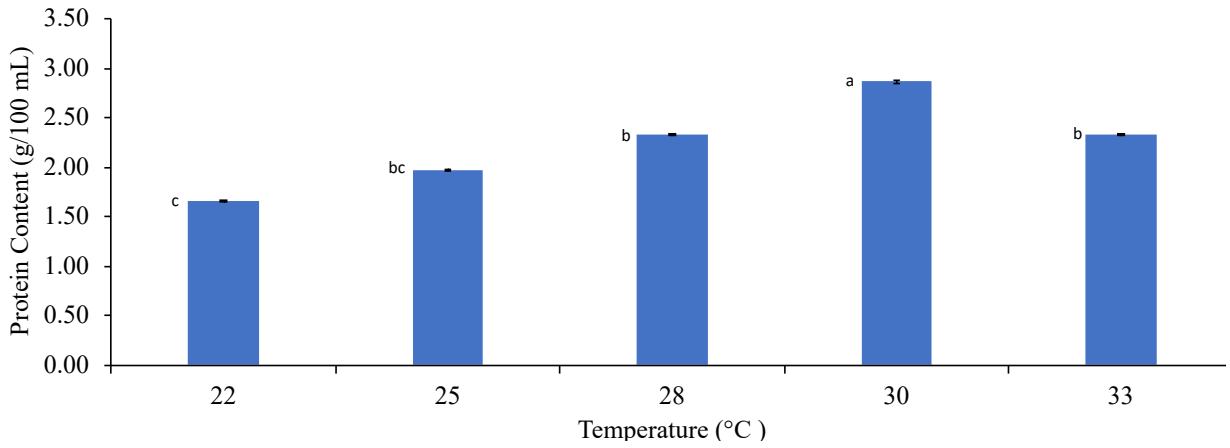


Figure 2. Protein content of beverages fermented at different temperatures (22°C, 25°C, 28°C, 30°C, 33°C), with mold (*A. oryzae*) and yeast added and fermented for a duration of 5 days, using Lowry's Protein Assay.

The beverage was then fermented at different durations, ranging from 2 days - 7 days, at 28°C with mold (*A. oryzae*) and yeast to maximize the protein content produced. The protein content of the beverage fermented for 7 days was the highest compared to the rest: 7 days (2.25 g/100 mL) > 3 days (2.20 g/100 mL) > 5 days (1.83 g/100 mL) > 2 days (1.77 g/100 mL) (**Fig. 3**). However, there was no significant difference between the protein contents of the beverages fermented for 7 days and the others. This observation showed that the duration of fermentation, ranging from 2 days - 7 days, do not have a significant effect on the protein content of the beverage.

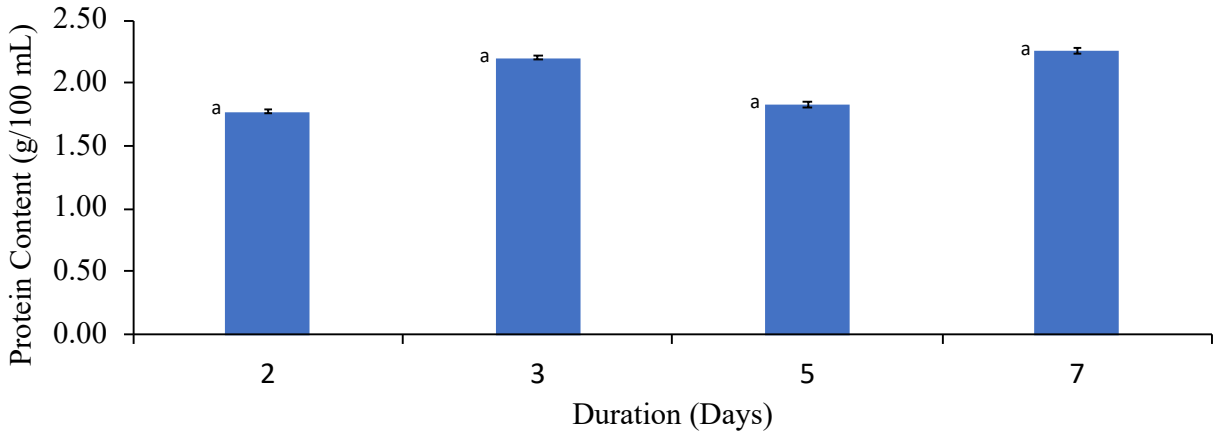


Figure 3. Protein content of beverages fermented at different durations (2, 3, 5, 7 days), with mold (*A. oryzae*) and yeast added and fermented at 28°C, using Lowry's Protein Assay.

Following these observations, it was concluded in order to optimize the protein content of the beverage, it was important to include the presence of yeast and for the fermentation process to be carried out at 30°C for 2 - 7 days.

Quantification of Bioactive Peptides

Parameters of fermentation were explored to further study the relationship between mold (*A. oryzae*), yeast and the concentrations of bioactive peptides. The bioactive peptides tested were VY, LSP, FR, YW, YPR, and LLPH.

Different types of starter cultures were used in the beverages and quantified for bioactive peptides using the HPLC/(+) ESI-SRM: control (no organisms), mold (*A. oryzae)*, yeast, and mold (*A. oryzae*) and yeast. The total concentration of bioactive peptides was found highest in the beverage with yeast: yeast (103 mg/100 mL) > control (94 mg/100 mL) > mold and yeast (91 mg/100 mL) > mold (68 mg/100 mL) (**Fig. 4)**.

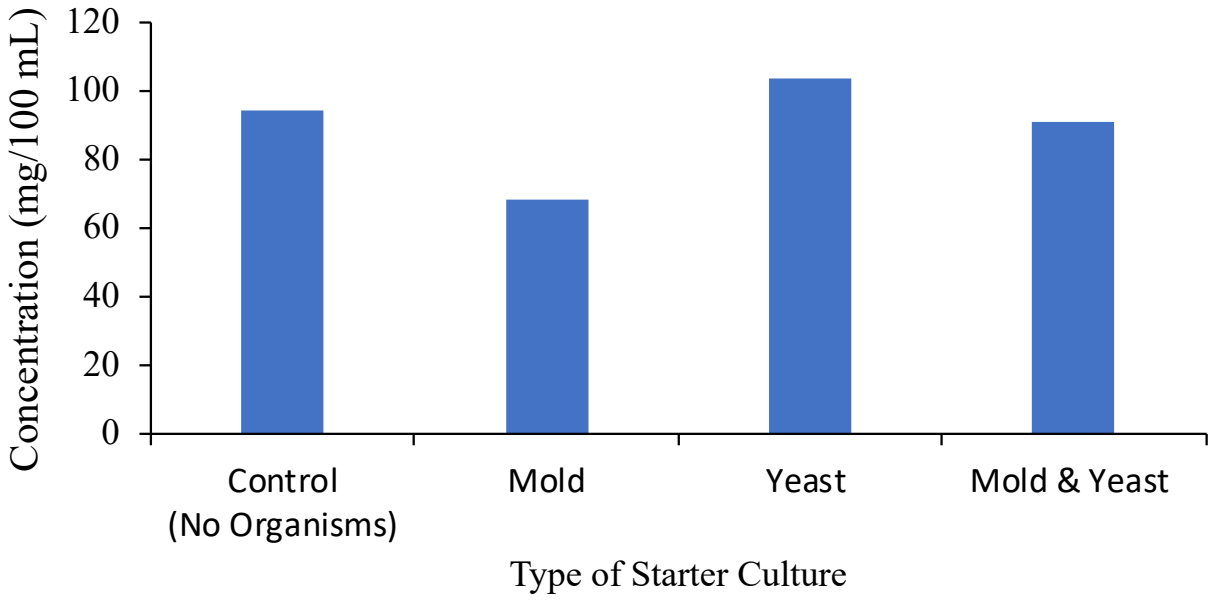


Figure 4. Total concentrations of bioactive peptides (VY, LSP, FR, YW, YPR, LLPH) in beverages made with different starter cultures (control, mold, yeast, yeast and mold) fermented at 28°C for a duration of 5 days, using HPLC/(+) ESI-SRM.

The bioactive peptide, VY, which functions as an angiotensin-converting enzyme (ACE) (Han & Xu, 2011), was found highest in the beverage that was the control, with no organisms added: control (60 mg/100 mL) > mold and yeast, and mold (30 mg/100 mL) > yeast (26 mg/100 mL) (**Fig. 5**). There was a significant difference between the VY content of the beverage that was the control, with no organisms added, and the others. This shows that the bioactive peptide VY may be present in rice grains, and mold (*A. oryzae*) and yeast may utilize or hydrolyze this peptide during fermentation.

The bioactive peptide, LSP, which also functions as an ACE (Han & Xu, 2011), was found highest in the beverage with mold and yeast: mold and yeast (30 mg/100 mL) > mold (28 mg/100 mL) > yeast (22 mg/100 mL) > control (21 mg/100 mL) (**Fig. 5**). There was a significant difference between the LSP content of the beverages with mold and yeast and mold, and the beverages with

yeast and the control. This shows that the presence of mold increases LSP content, indicating that mold (*A. oryzae*) may secrete enzymes that lead to the production of LSP.

The bioactive peptide, FR, which functions as a dipeptidyl-aminopeptidase IV inhibitor and an antioxidant (Han & Xu, 2011), was found highest in the beverage with yeast: yeast (55 mg/100 mL) > mold and yeast (31 mg/100 mL) > control (13 mg/100 mL) > mold (10 mg/100 mL) (**Fig. 5**). There was a significant difference between the FR content of the beverage with yeast and the others. In addition, there was a significant difference between the FR content of the beverages with mold and yeast and the others. However, there was no significant difference between the beverages with mold and the control, which had no organisms added. This shows that the presence of yeast increases the FR content, indicating that it may secrete enzymes that lead to the production of FR. On the other hand, the presence of mold decreases the FR content, indicating that it may utilize or secrete enzymes that lead to the decrease in FR content.

The bioactive peptide, YW, which also functions as an ACE (Han & Xu, 2011), was found in trace amounts in all of the beverages (**Fig. 5**). However, its minute amounts of below 0.1% were too small to be considered significant. Other bioactive peptides, such as YPR and LLPH, were tested and results came out negative for all beverages (**Fig. 5**). Following this observation, parameters of fermentation were further explored to study the relationship between mold (*A. oryzae*), yeast, and the concentrations of bioactive peptides.

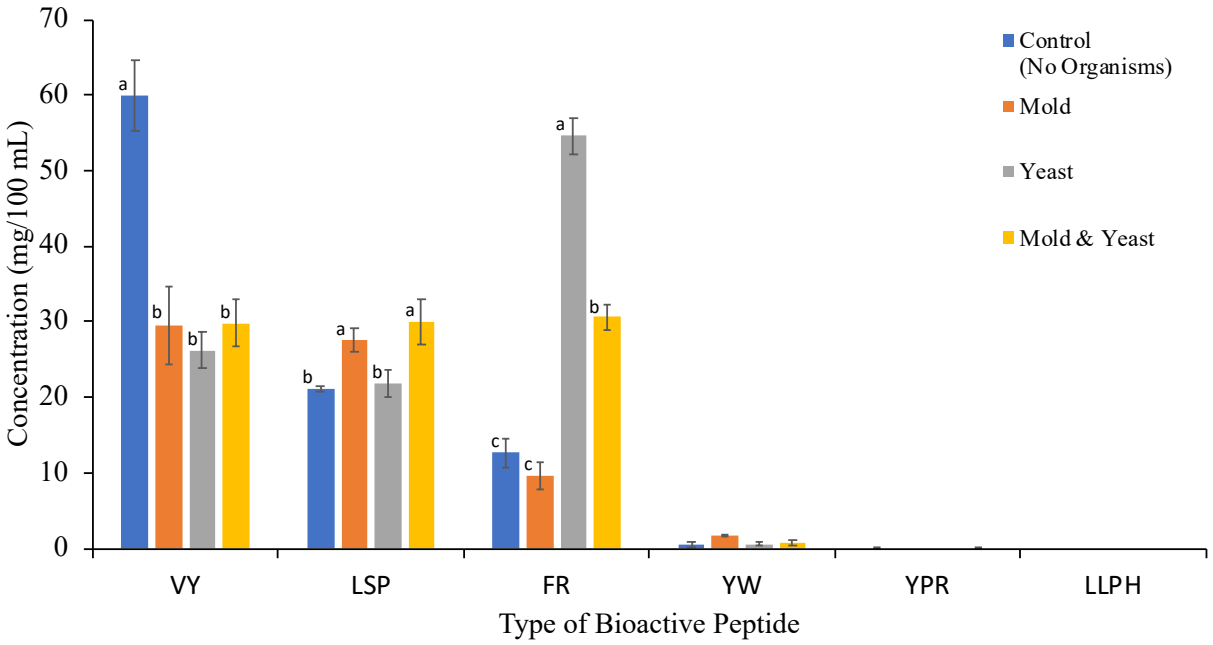


Figure 5. Concentrations of bioactive peptides (VY, LSP, FR, YW, YPR, and LLPH) in beverages made with different starter cultures (control, mold (*A. oryzae*), yeast, mold (*A. oryzae*) and yeast) fermented at 28°C for a duration of 5 days, using HPLC/(+) ESI-SRM.

The rice beverages were then fermented at different temperatures, ranging from 22°C - 33°C, with mold and yeast, and quantified for bioactive peptides using HPLC/(+) ESI-SRM. The total concentration of bioactive peptides was found highest in the beverage fermented at 22°C: 22°C (106 mg/100 mL) > 25°C (101 mg/100 mL) > 28°C (91 mg/100 mL) > 33°C (85 mg/100 mL) > 30°C (84 mg/100 mL) (**Fig. 6**).

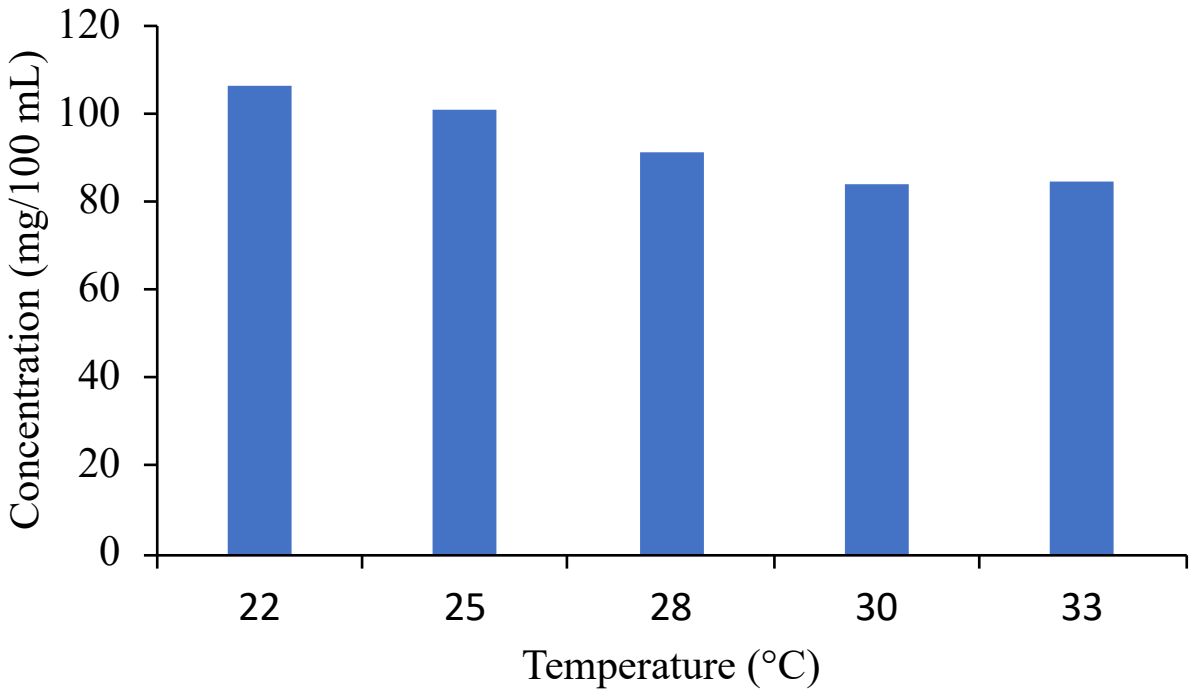


Figure 6. Total concentrations of bioactive peptides (VY, LSP, FR, YW, YPR, LLPH) in beverages fermented at different temperatures (22°C, 25°C, 28°C, 30°C, 33°C), with mold (*A. oryzae*) and yeast and fermented for a duration of 5 days, using HPLC/(+) ESI-SRM.

The bioactive peptide, VY, was found highest in the beverage that was fermented at 30°C: 30°C (33 mg/100 mL) > 28°C (30 mg/100 mL) > 33°C (28 mg/100 mL) > 25°C (27 mg/100 mL) > 22°C (26 mg/100 mL) (**Fig. 7**). There was a significant difference between the VY content of the beverages that were fermented at 28°C, 30°C and 33°C, and the beverages that were fermented at 22°C and 25°C. This shows that the bioactive peptide VY was produced at a higher rate at temperatures of 28°C - 33°C, in comparison to temperatures of 22 - 25°C, with the highest rate recorded at 30°C.

The bioactive peptide, LSP, was found highest in the beverage that was fermented at 25°C: 25°C (36 mg/100 mL) > 28°C (30 mg/100 mL) > 22°C and 33°C (29 mg/100 mL) > 30°C (24 mg/100 mL) (**Fig. 7**). There was a significant difference between the LSP content of the beverage that was

fermented at 25°C and the beverage that was fermented at 30°C. However, there is no significant difference in LSP content of the beverages that were fermented at 22°C, 25°C, 28°C and 33°C. Neither was there any significant difference in LSP content of the beverages that were fermented at 22°C, 28°C, 30°C and 33°C. This shows that the bioactive peptide LSP was produced at a higher rate at 25°C in comparison to 30°C.

The bioactive peptide, FR, was found highest in the beverage that was fermented at 22°C: 22°C (51 mg/100 mL) > 25°C (37 mg/100 mL) > 28°C (31 mg/100 mL) > 33°C (27 mg/100 mL) > 30°C (26 mg/100 mL) (**Fig. 7**). There was a significant difference between the FR content of the beverage that was fermented at 22°C and the others. There was also a significant difference between the FR content of the beverage that was fermented at 25°C, and the beverages that were fermented at 30°C and 33°C. However, there are no significant differences between the beverages that were fermented at 25°C and 28°C. Neither was there any significant differences between the beverages that were fermented at 28°C, 30°C, and 33°C. This shows that the bioactive peptide FR was produced at a higher rate at 22°C in comparison to 25°C - 33°C.

The bioactive peptide, YW, was found in trace amounts in all of the beverages (**Fig. 7**). However, its minute amounts of below 0.1% were too small to be considered significant. Other bioactive peptides, such as YPR and LLPH, were tested and results came out negative for all beverages (**Fig. 7**). Following this observation, the duration of fermentation was explored to further study the relationship between mold (*A. oryzae*), yeast and the concentrations of bioactive peptides.

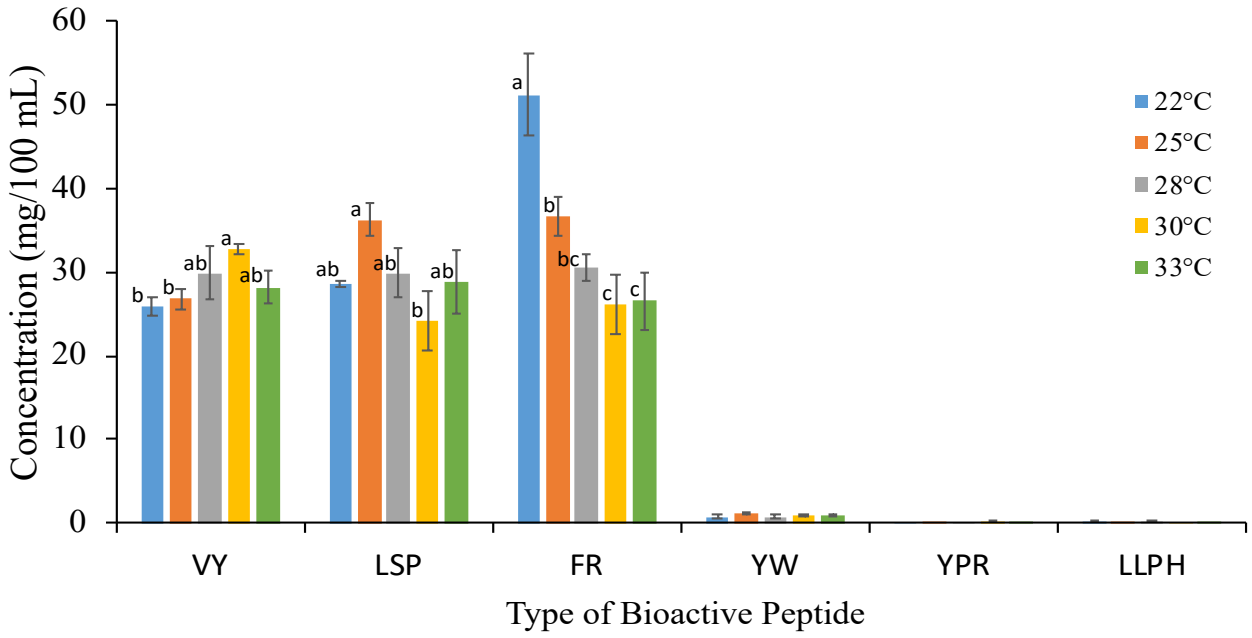


Figure 7. Concentrations of bioactive peptides (VY, LSP, FR, YW, YPR, and LLPH) in beverages fermented at different temperatures (22°C, 25°C, 28°C, 30°C, 33°C), with mold (*A. oryzae*) and yeast and fermented for a duration of 5 days, using HPLC/(+) ESI-SRM.

The beverage was then fermented at different durations, ranging from 2 days to 7 days, at 28°C with mold (*A. oryzae*) and yeast, and quantified for bioactive peptides using HPLC/(+) ESI-SRM. The total concentration of bioactive peptides was found highest in the beverage fermented for 2 days: 2 days (119 mg/100 mL) > 3 days (113 mg/100 mL) > 5 days and 7 days (97 mg/100 mL) (**Fig. 8**).

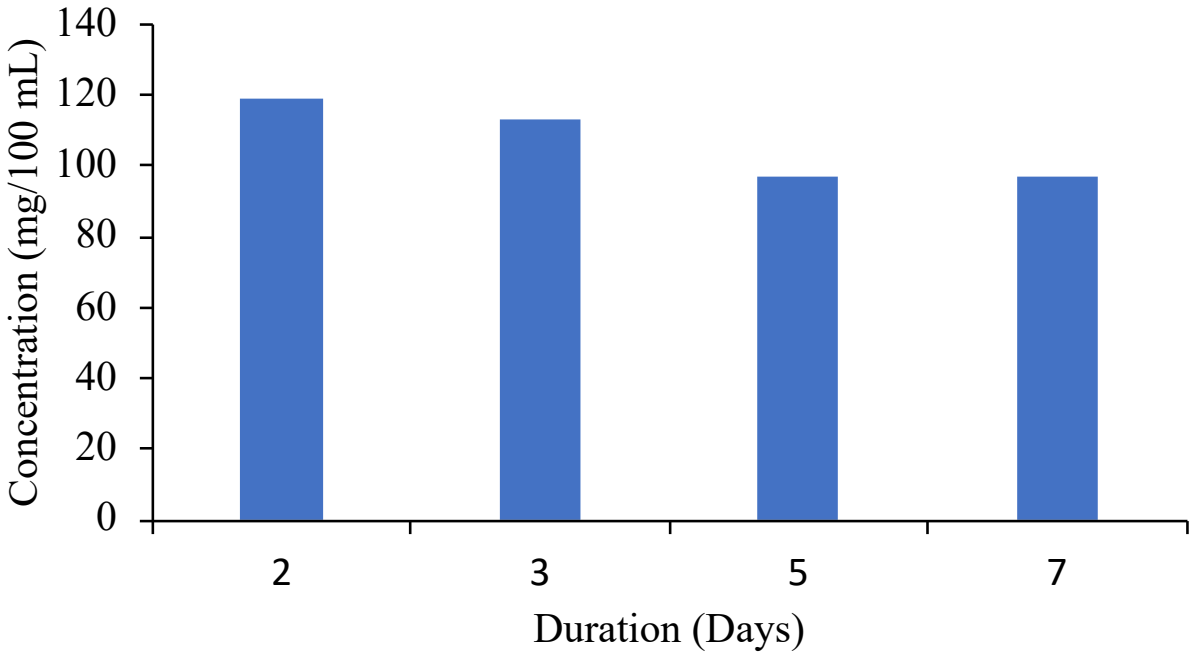


Figure 8. Total concentrations of bioactive peptides (VY, LSP, FR, YW, YPR, LLPH) in beverages fermented at different durations (2, 3, 5, 7 days), with mold (*A. oryzae*) and yeast and fermented at 28°C, using HPLC/(+) ESI-SRM.

The bioactive peptide, VY, was found highest in the beverage that was fermented for 2 days: 2 days (91 mg/100 mL) > 3 days (71 mg/100 mL) > 5 days (35 mg/100 mL) > 7 days (30 mg/100 mL) (**Fig. 9**). There was a significant difference between the VY content of the beverages that were fermented for 2 days and the others. There was also a significant difference between the VY content of the beverages that were fermented for 3 days and the others. However, there is no significant difference between the VY content of the beverages that were fermented for 5 days and 7 days. This shows that the bioactive peptide VY was produced at the highest rate when the beverage was left to ferment for 2 days.

The bioactive peptide, LSP, was found highest in the beverage that was fermented at 7 days: 7 days (29 mg/100 mL) > 5 days (27 mg/100 mL) > 3 days (21 mg/100 mL) > 2 days (13 mg/100 mL) (**Fig. 9**). There was a significant difference between the LSP content of the beverages that

were fermented for 5 and 7 days and the beverages that were fermented for 2 and 3 days. There was also a significant difference between the LSP content of the beverage that was fermented for 3 days and the beverage that was fermented for 2 days. However, there is no significant difference in LSP content of the beverages that were fermented for 5 days and 7 days. This shows that the bioactive peptide LSP was produced at the highest rate when the beverage is left to ferment for 7 days.

The bioactive peptide, FR, was found highest in the beverage that was fermented at 7 days: 7 days (36 mg/100 mL) > 5 days (34 mg/100 mL) > 3 days (21 mg/100 mL) > 2 days (15 mg/100 mL) (**Fig. 9**). There was a significant difference between the FR content of the beverages that were fermented for 5 and 7 days and the beverages that were fermented for 2 and 3 days. However, there was no significant difference in FR content of the beverages that were fermented for 5 days and 7 days. In addition, there was also no significant difference in FR content of the beverages that were fermented for 2days and 3 days. This shows that the bioactive peptide FR was produced at the highest rate when the beverage is left to ferment for 5 - 7 days.

The bioactive peptide, YW, was found in trace amounts in all of the beverages (**Fig. 9**). However, its minute amounts of below 0.1% were too small to be considered significant. Other bioactive peptides, such as YPR and LLPH, were tested and results came out negative for all beverages (**Fig. 9**).

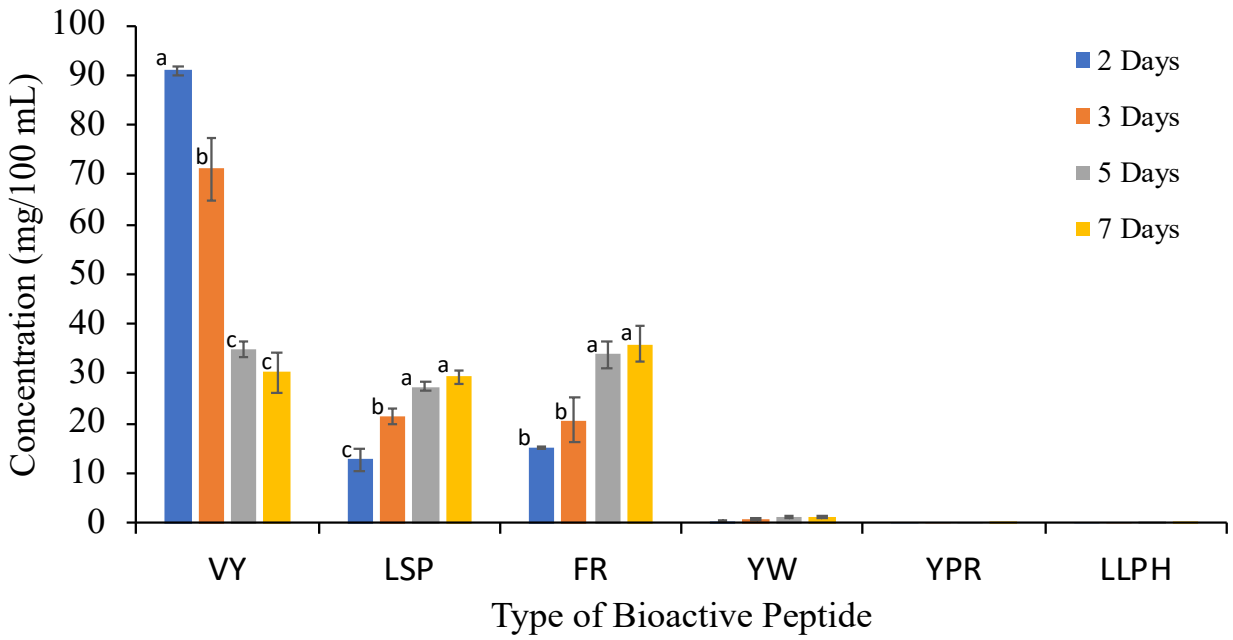


Figure 9. Concentrations of bioactive peptides (VY, LSP, FR, YW, YPR, and LLPH) in beverages fermented at different durations (2, 3, 5, 7 days), with mold (*A. oryzae*) and yeast and fermented at 28°C, using HPLC/(+) ESI-SRM.

Following these observations, it was concluded that in order to optimize and achieve the most distributed concentrations of bioactive peptides in the beverage, it was important to include both the presences of mold (*A. oryzae*) and yeast, and for the fermentation to be carried out 28°C - 33°C for 5 - 7 days.

Quantification of Vitamin B-Complex

Parameters of fermentation were also explored to further study the relationship between mold (*A. oryzae*), yeast and the levels of vitamin B-complex. The vitamin B-complex tested were B1, B3, B5, and B6.

Different types of starter cultures were used in the beverages and quantified for vitamin B-complex using the LCMS: control (no organisms), mold (*A. oryzae*), yeast, and mold (*A. oryzae*) and yeast.

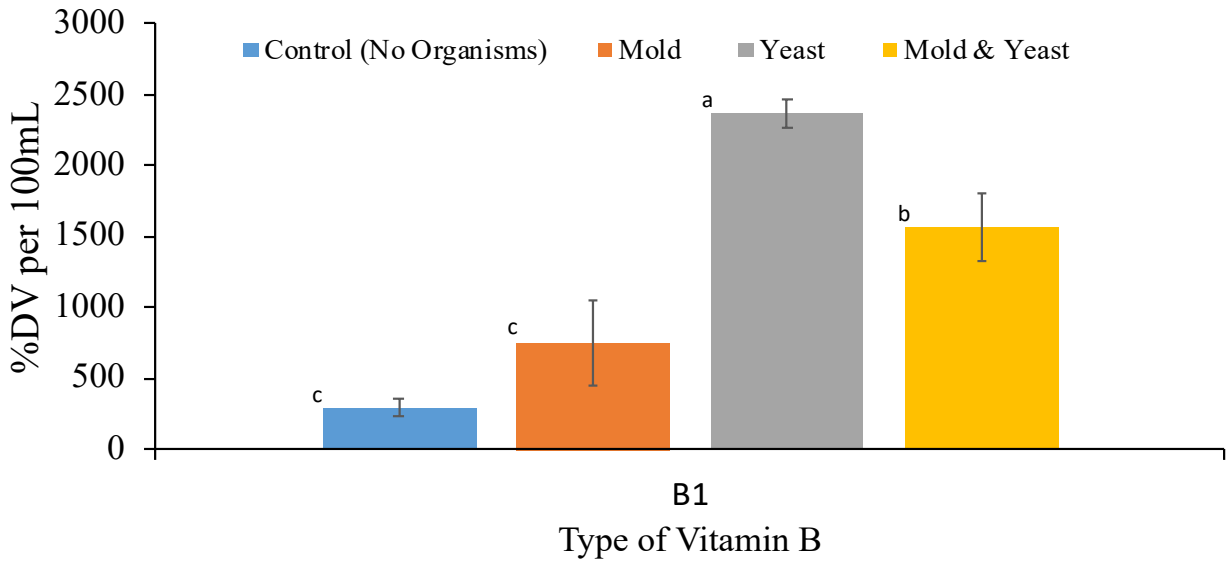
Vitamin B1 was found highest in the beverage with yeast: yeast (2368% DV/100 mL) > mold and yeast (1562% DV/100 mL) > mold (748% DV/100 mL) > control (293% DV/100 mL) (**Fig. 10**). There was a significant difference between the vitamin B1 contents of the beverages with yeast and the others. Also, there was a significant difference between the vitamin B1 contents of the beverages with mold and yeast and the others. However, there was no significant difference between the beverages with mold and the control, which had no organisms added. This shows that the presence of yeast increased the vitamin B1 content of the beverages, the highest observed in comparison to the vitamin B1 content of the beverages with mold and yeast.

Vitamin B3 was found highest in the beverage with mold and yeast: mold and yeast (11% DV/100 mL) > yeast (9% DV/100 mL) > control (3% DV/100 mL) > mold (2% DV/100 mL) (**Fig. 10**). There was a significant difference between the vitamin B3 contents of the beverages with yeast and mold and yeast, and the beverages with mold and the control. However, there was no significant difference between the vitamin B3 content of the beverage with mold and yeast and the beverage with yeast. Also, there was no significant difference between the vitamin B3 content of the beverage with mold and the control. This shows that vitamin B3 may be synthesized by yeast during fermentation.

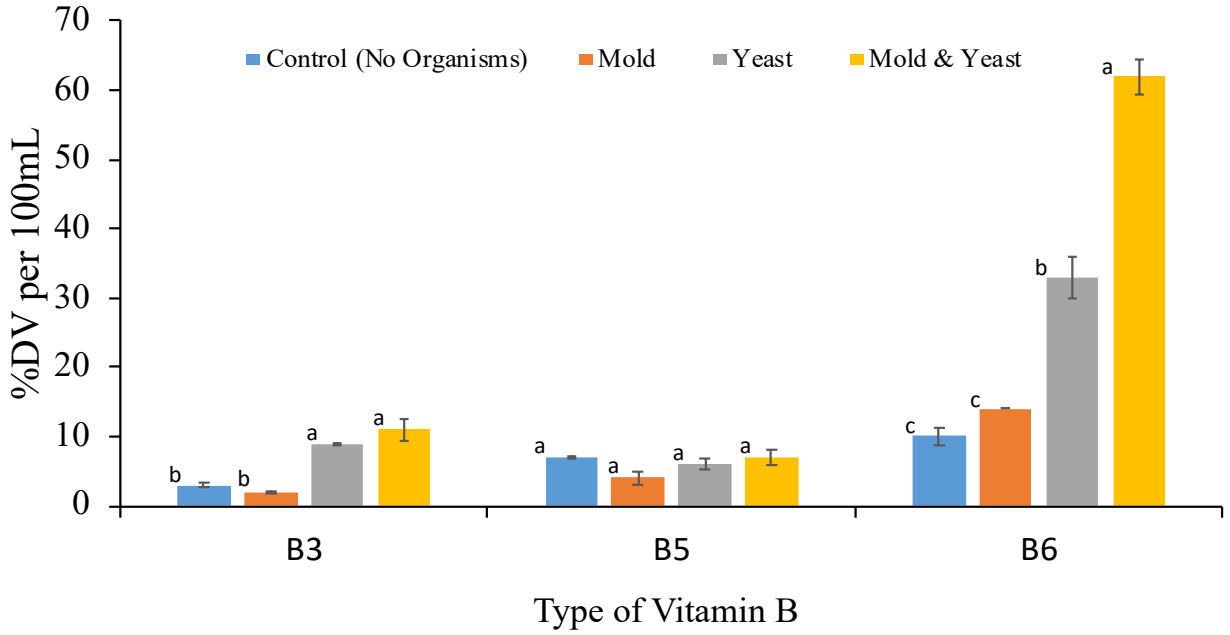
Vitamin B5 was found highest in the beverage with mold and yeast and the control: mold and yeast and the control (7% DV/100 mL) > yeast (6% DV/100 mL) > mold (4% DV/100 mL) (**Fig. 10**). However, there was no significant difference in vitamin B5 content between the beverages. This may indicate that the neither the presence of yeast nor mold affected the content of vitamin B5.

Vitamin B6 was found highest in the beverage with mold and yeast: mold and yeast (62% DV/100 mL) > yeast (33% DV/100 mL) > mold (14% DV/100 mL) > control (10% DV/100 mL) (**Fig. 10**).

There was a significant difference between the vitamin B6 content of the beverage with mold and yeast and the others. In addition, there was a significant difference between the vitamin B6 contents of the beverages with yeast and the others. However, there was no significant difference between the beverage with mold and the control, which had no organisms added. This shows that the presence of yeast increases the vitamin B6 content of the beverages, but the presence of both mold and yeast further increases the vitamin B6 content of the beverage, more than when there was only yeast added. Following this observation, parameters of fermentation were explored to further study the relationship between mold (*A. oryza*), yeast and the levels of vitamin B-complex.



A



B

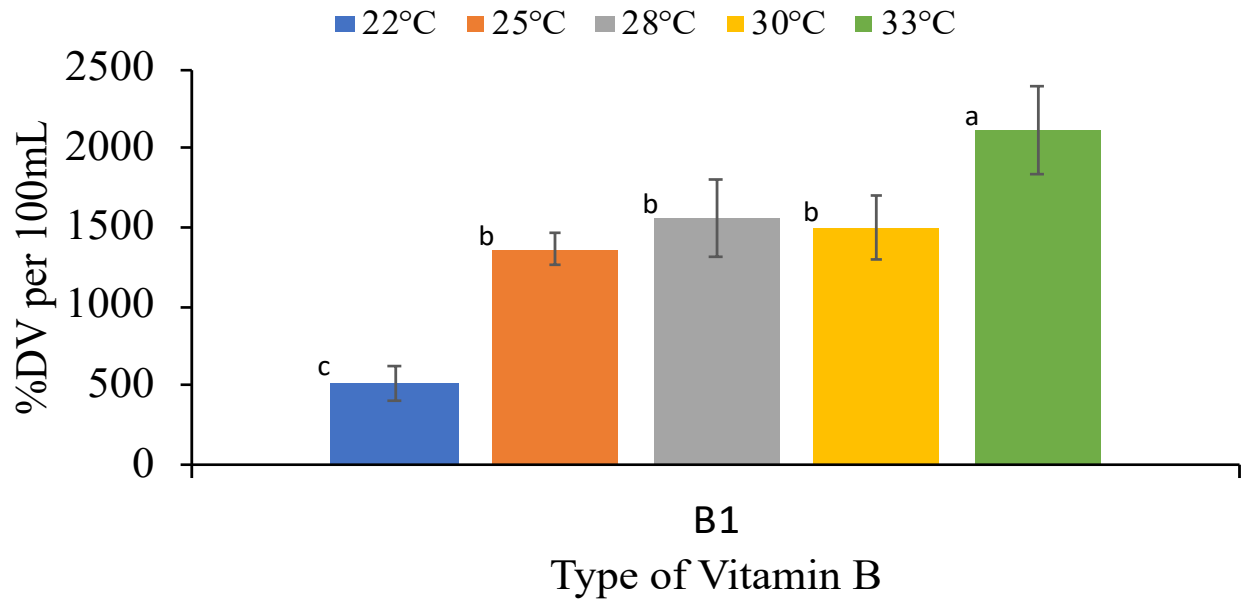
Figure 10. % Daily values of vitamin B1 (**A**), B3, B5 and B6 (**B**) per 100 mL of beverages made with different starter cultures (control, mold (*A. oryzae*), yeast, mold (*A. oryzae*) and yeast) fermented at 28°C for a duration of 5 days, using HPLC/(+) ESI-SRM.

The rice beverages were then fermented at different temperatures, ranging from 22°C - 33°C, with mold and yeast, and quantified for vitamin B-complex using LCMS. Vitamin B1 was found highest in the beverage that was fermented at 33°C: 33°C (2116% DV/100 mL) > 28°C (1562% DV/100 mL) > 30°C (1496% DV/100 mL) > 25°C (1363% DV/100 mL) > 22°C (512% DV/100 mL) (**Fig. 11**). There was a significant difference between the vitamin B1 content of the beverage that was fermented at 33°C and the others, which were fermented at 22°C, 25°C, 30°C and 33°C. In addition, there was no significant difference between the beverages that were fermented at 25°C, 28°C, and 30°C. This shows that 33°C was the most favorable fermentation temperature for vitamin B1 production in the presence of mold and yeast.

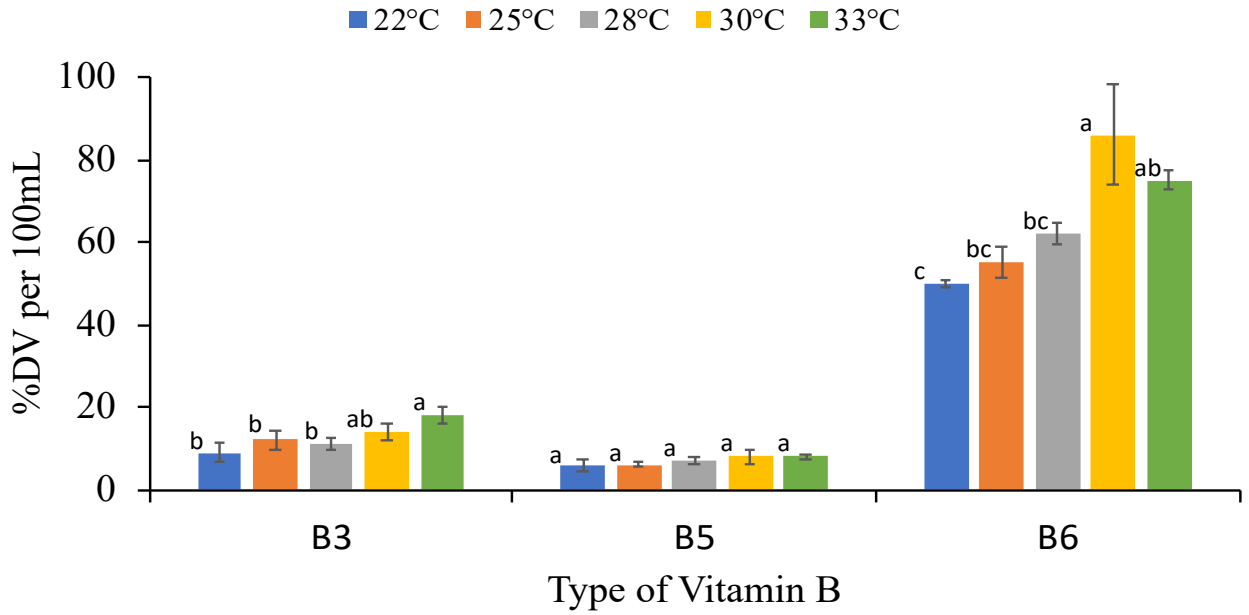
Vitamin B3 was found highest in the beverage that was fermented at 33°C: 33°C (18% DV/100 mL) > 30°C (14% DV/100 mL) > 25°C (12% DV/100 mL) > 28°C (11% DV/100 mL) > 22°C (9% DV/100 mL) (**Fig. 11**). There was a significant difference between the vitamin B3 content of the beverage that was fermented at 33°C and the beverages that were fermented at 22°C, 25°C and 28°C. However, there was no significant difference between the beverages that were fermented at 30°C and 33°C. Also, there was no significant difference between the beverages that were fermented at 22°C, 25°C, 28°C and 30°C. This shows that 33°C was the most favorable fermentation temperature for vitamin B3 production in the presence of mold and yeast.

Vitamin B5 was found highest in the beverage that was fermented at 30°C and 33°C: 30°C & 33°C (8% DV/100 mL) > 28°C (7% DV/100 mL) > 22°C & 25°C (6% DV/100 mL) (**Fig. 11**). However, there was no significant difference between the beverages. This shows that the temperatures, ranging from 22°C to 33°C, does not significantly influence the production of vitamin B5 in the presence of mold and yeast.

Vitamin B6 was found highest in the beverage that was fermented at 30°C: 30°C (86% DV/100 mL) > 33°C (75% DV/100 mL) > 28°C (62% DV/100 mL) > 25°C (55% DV/100 mL) > 22°C (50% DV/100 mL) (**Fig. 11**). There was a significant difference between the vitamin B6 content of the beverage that was fermented at 30°C and the beverages that were fermented at 22°C, 25°C and 28°C. However, there was no significant difference between the beverages that were fermented at 30°C and 33°C. Also, there was no significant difference between the beverages that were fermented at 22°C, 25°C, 28°C and 30°C. This shows that 30°C was the most favorable fermentation temperature for vitamin B6 production in the presence of mold and yeast. Following this observation, the duration of fermentation was explored to further study the relationship between mold (*A. oryzae*), yeast and the levels of vitamin B-complex.



A



B

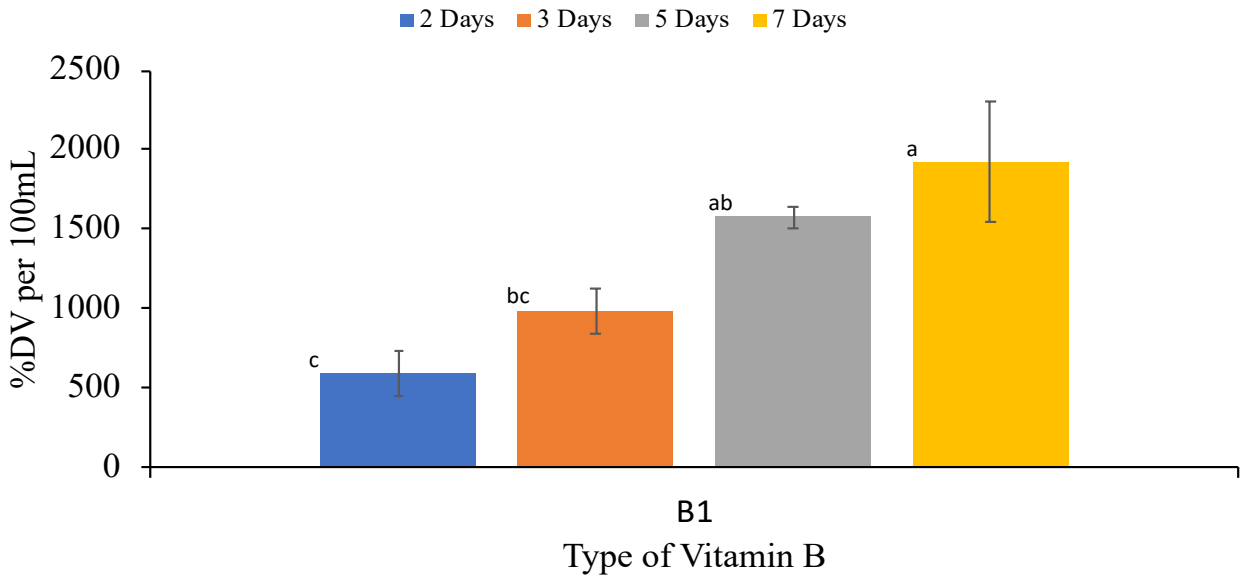
Figure 11. % Daily values of vitamin B1 (**A**), B3, B5 and B6 (**B**) per 100 mL of beverages fermented at different temperatures (22°C, 25°C, 28°C, 30°C, 33°C), with mold (*A. oryzae*) and yeast added and fermented for a duration of 5 days, using HPLC/(+) ESI-SRM.

The beverage was then fermented at different durations, ranging from 2 days - 7 days, at 28°C with mold (*A. oryzae*) and yeast, and quantified for vitamin B-complex using HPLC/(+) ESI-SRM. Vitamin B1 was found highest in the beverage that was fermented for 7 days: 7 days (1923% DV/100 mL) > 5 days (1574% DV/100 mL) > 3 days (984% DV/100 mL) > 2 days (594% DV/100 mL) (**Fig. 12**). There was a significant difference between the vitamin B1 content of the beverages that were fermented for 5 and 7 days, and the beverage that were fermented for 2 days. In addition, there was no significant difference between the beverages that were fermented for 2 and 3 days. There was also no significant difference between the beverages that were fermented for 3 and 5 days. On top of that, there was also no significant difference between the beverages that were fermented for 5 and 7 days. This shows that 5 - 7 days were the most favorable fermentation duration for vitamin B1 production in the presence of mold and yeast.

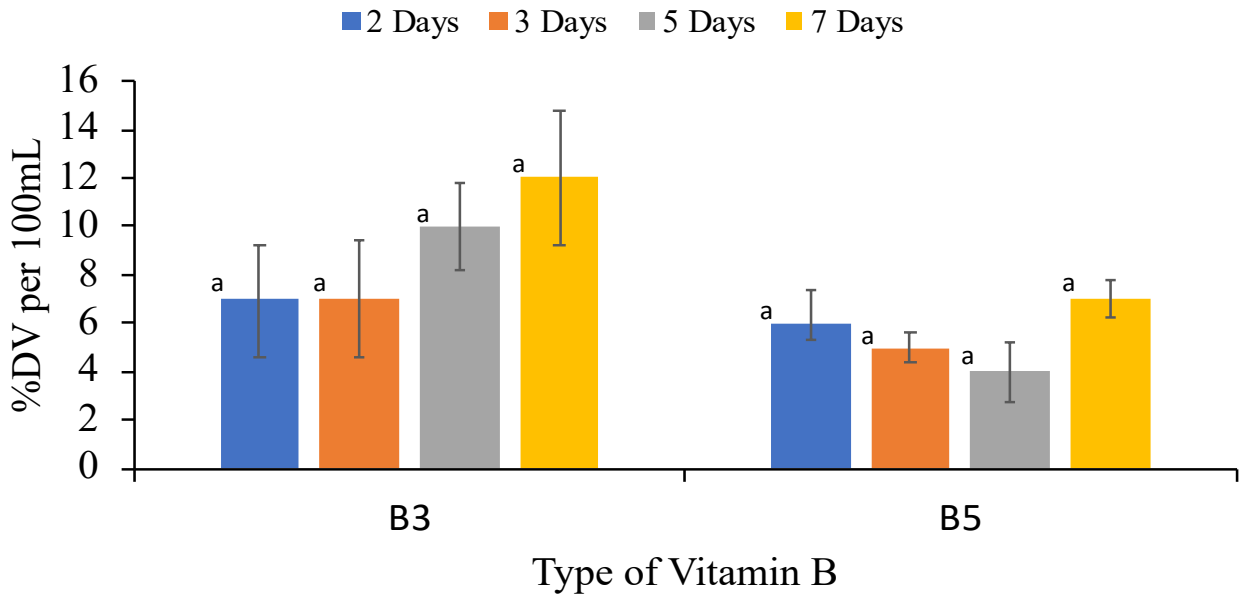
Vitamin B3 was found highest in the beverage that was fermented for 7 days: 7 days (12% DV/100 mL) > 5 days (10% DV/100 mL) > 2 days and 3 days (7% DV/100 mL) (**Fig. 12**). However, there was no significant difference between the beverages that were fermented for 2, 3, 5 and 7 days. This shows that the fermentation duration from 2 - 7 days does not significantly affect the vitamin B3 production in the presence of mold and yeast.

Vitamin B5 was found highest in the beverage that was fermented for 7 days: 7 days (7% DV/100 mL) > 2 days (6% DV/100 mL) > 3 days (5% DV/100 mL) > 5 days (4% DV/100 mL) (**Fig. 12**). However, there was no significant difference between the beverages that were fermented for 2, 3, 5 and 7 days. This shows that the fermentation duration from 2 - 7 days does not significantly affect the vitamin B5 production in the presence of mold and yeast.

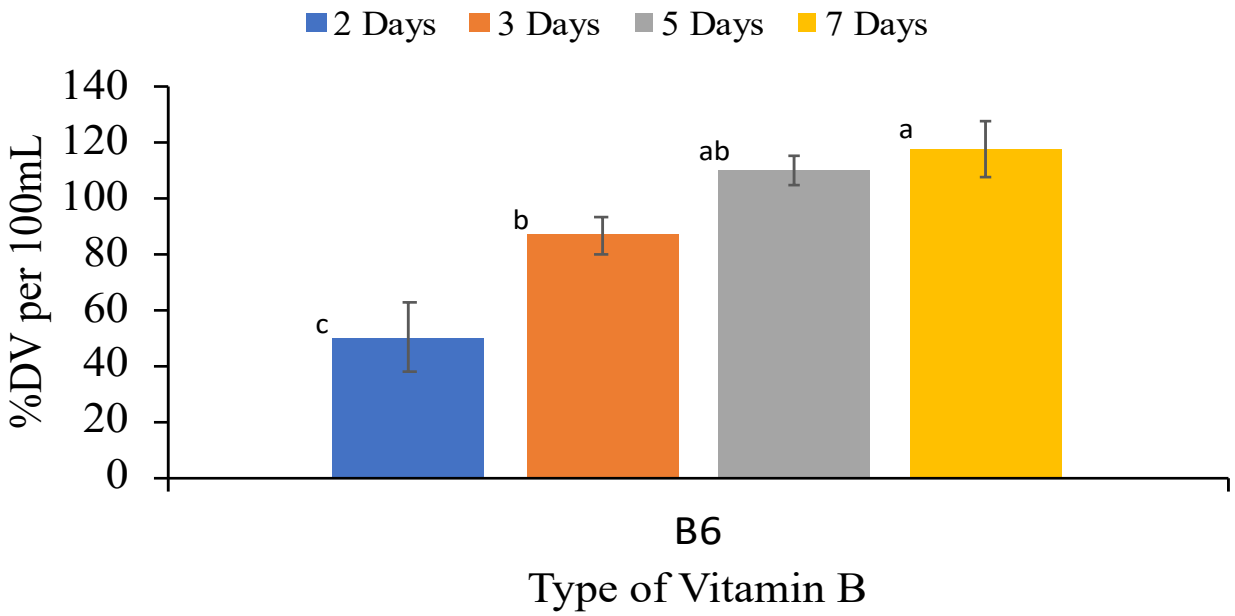
Vitamin B6 was found highest in the beverage that was fermented for 7 days: 7 days (118% DV/100 mL) > 5 days (110% DV/100 mL) > 3 days (87% DV/100 mL) > 2 days (50% DV/100 mL) (**Fig. 12**). There was a significant difference between the vitamin B6 content of the beverages that were fermented for 5 and 7 days, and the beverages that were fermented for 2 and 3 days. However, there was no significant difference between the beverages that were fermented for 5 and 7 days. In addition, there was also no significant difference between the beverages that were fermented for 3 and 5 days. This shows that 5 - 7 days were the most favorable fermentation duration for vitamin B6 production in the presence of mold and yeast.


2 Days
3 Days
5 Days
7 Days
2500
2000
1500
1000
500
0
%DV per 100mL
c
bc
ab
a
B1
Type of Vitamin B

A


2 Days
3 Days
5 Days
7 Days
16
14
12
10
8
6
4
2
0
%DV per 100mL
a
a
a
a
a
a
a
a
B3
B5
Type of Vitamin B

B



C

Figure 12. % Daily values of vitamin B1 (**A**), B3, B5 (**B**) and B6 (**C**) per 100 mL of beverages fermented at different durations (2, 3, 5, 7 days), with mold (*A. oryzae*) and yeast added and fermented at 28°C, using HPLC/(+) ESI-SRM.

Following these observations, it was concluded that in order to optimize and achieve the most distributed concentrations of vitamins B1, B3, B5 and B6 in the beverage, it was important to include both the presences of mold (*A. oryzae*) and yeast, and for the fermentation to be carried out 30°C - 33°C for 5 - 7 days.

CHAPTER 4

CONCLUSION

This study quantified protein, vitamin B-complex and bioactive peptides imparted by *A. oryzae* and yeast activities on rice grains, and gave insight on how different starter cultures, fermentation temperatures and durations affect these concentrations in an attempt to understand the relationship between *A. oryzae*, yeast and the concentrations of protein, vitamin B-complex and bioactive peptides, to capture these beneficial elements in a nutritious beverage.

The presence of *A. oryzae* was important in significantly increasing concentrations of bioactive peptides LSP, YW, and vitamin B6, whilst the presence of yeast was important in significantly increasing concentrations of protein, bioactive peptide FR, vitamin B1, vitamin B3, and vitamin B6. *A. oryzae* and yeast was shown to have a symbiotic relationship, especially in the synthesis of vitamin B6.

Fermenting the beverage at 22°C worked best at achieving the highest concentration of bioactive peptide FR. On the other hand, fermenting the beverage at 30°C saw the highest concentrations of protein and vitamin B6. In addition, fermenting the beverage at 33°C yielded the highest concentration of vitamin B1. There are other nutrients, such as bioactive peptide VY and vitamin B3, that saw its increased concentrations over a range of temperatures. However, fermentation temperatures of 22°C - 33°C does not seem to have significant effect on the concentrations of bioactive peptides LSP and YW, and vitamin B5.

Fermenting the beverage for 2 days worked best at achieving the highest concentration of bioactive peptide VY. Other nutrients, such as bioactive peptides LSP, FR, and YW, vitamin B1 and vitamin

B6, saw its increased concentrations over a range of durations. However, fermentation durations of 2 - 7 days do not seem to have significant effect on the concentrations of protein, vitamin B3, and vitamin B5.

Bioactive peptides YPR and LLPH are not synthesized by *A. oryzae* or yeast. In addition, vitamin B5 seem to be naturally found in the beverage from the rice grains as neither the presence of *A. oryzae* nor yeast contributed significantly to its concentration.

Hence, it is important to include both *A. oryzae* and yeast in the making of the beverage to maximize its content in protein, bioactive peptides and vitamin B-complex. Also, fermenting the beverage at 28°C for 5 days would yield the most distributed nutrient content. Further studies should be done to investigate the symbiotic relationship between *A. oryzae* and yeast and understand the mechanisms that led to the synthesis of essential nutrients such as bioactive peptides and vitamin B-complex. The insights from this study and future work on using *A. oryzae* and yeast to enhance nutrients in our foods may be the key to naturally developing a nutrient-dense product.

REFERENCES

Chakrabarti, S., Guha, S., & Majumder, K. (2018). Food-Derived Bioactive Peptides in Human Health: Challenges and Opportunities. *Nutrients*, *10*(11), 1738. doi:10.3390/nu10111738

Han, F. L., & Xu, Y. (2011). Identification of Low Molecular Weight Peptides in Chinese Rice Wine (Huang Jiu) by UPLC-ESI-MS/MS. *Journal of the Institute of Brewing, 117*(2), 238-250. doi:10.1002/j.2050-0416.2011.tb00467.x

Kitts, D., & Weiler, K. (2003). Bioactive Proteins and Peptides from Food Sources. Applications of Bioprocesses used in Isolation and Recovery. *Current Pharmaceutical Design, 9*(16), 1309-1323. doi:10.2174/1381612033454883

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*(9), 945-960. doi:10.1016/j.idairyj.2005.10.012

Ogrydziak, D. M. (1993). Yeast Extracellular Proteases. *Critical Reviews in Biotechnology, 13*(1), 1-55. doi:10.3109/07388559309069197

Stanton, C., Ross, R. P., Fitzgerald, G. F., & Sinderen, D. V. (2005). Fermented functional foods based on probiotics and their biogenic metabolites. *Current Opinion in Biotechnology*, 16(2), 198-203. doi:10.1016/j.copbio.2005.02.008

"Vitamin B Complex." (2005). Gale Encyclopedia of Nursing and Allied Health. Retrieved April 26, 2019 from Encyclopedia.com: https://www.encyclopedia.com/medicine/encyclopedias-almanacs-transcripts-and-maps/vitamin-b-complex-0

Zanutto-Elgui, M. R., Vieira, J. C., Prado, D. Z., Buzalaf, M. A., Padilha, P. D., Oliveira, D. E., & Fleuri, L. F. (2019). Production of milk peptides with antimicrobial and antioxidant properties through fungal proteases. *Food Chemistry*, 278, 823-831. doi:10.1016/j.foodchem.2018.11.119