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Isolation and Identification of Cholesterol Lowering Probiotic Yeast from Palm Raffia (*Raffia mambillensis*) Wine

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Authors' contributions

This work was carried out in collaboration between all authors. Authors EFTN, ZNF and BT designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BTF, GTN, EFTN and BT managed the analyses of the study. Authors BT and DMS managed the literature searches. Authors ZNF and FTM supervised the work. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of the present work was to study the cholesterol lowering effects of probiotic yeasts isolated from raffia (*Raffia mambillensis*) wine.
Study Design: Collection of fermented raffia wine samples, culture, isolation, identification on culture media and selection of cholesterol lowering strain *in vivo* and *in vitro*.
Place and Duration of Study: This study was conducted in the Teaching Laboratory of Life Science, Department of Biochemistry, University of Buea from February 2016 to July 2016.

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Methodology: Six different palm wine samples were collected and used for selection of yeasts isolates on sabouraud Dextrose Agar using pour plate method and those with acid, bile tolerance and cholesterol assimilation *in vitro* selected. Strains were identified using the API 20C AUX and their cholesterol lowering effect on wistar albino rats studied. The *in vivo* experiment was carried out for four weeks by oral gavage; dose level 10^8-10^{10} CFU/ ml. The administered volume of each dose was 1.0 ml/kg body weight/day. After the feeding trial, the rats were dissected and serum lipid profile analyzed using biochemical kits.

Results: From six raffia wine samples, twenty eight yeasts isolates were selected based on their morphology; twelve isolates showed resistance to low pH and bile salts. Among them four isolates were able to assimilate at least 50% cholesterol in culture media and were biochemically characterized using the API® 20C AUX BioMerieux kit, which revealed 94.9%, 95.3%, 51.5% and 92.2%, homology of 1R27, 1W29, 2R29 and 3W29 with *Saccharomyces cerevisiae, Candida famata, Cryptococcus neoformans and Candida famata respectively*. Based on their best *in vitro* assimilation, strain 1R27 and 3W29 were further used for *in vivo* tests. Both strains showed evidence of their capacity *in vivo* to adhere to epithelial intestine-derived cells, reduce lipidemia (tryglyceride, total cholesterol and LDL) and increase HDL. All the two strains confirm their cholesterol lowering properties *in vivo*. The probiotic strains *Saccharomyces cerevisiae* was the most efficient on reduction of the levels of total cholesterol, LDL and VLDL in the rats blood serum compared to *Candida famata.*

Conclusion: These probiotic strains can therefore be used to improve the product quality and develop added value to dairy products. Hypercholesterolemia is a major risk factor for coronary artery disease and probiotics can then be suggested as tools to manage elevated cholesterol levels.

Keywords: Probiotic yeasts; cholesterol; lipidemia; bile tolerance; acid tolerance.

1. INTRODUCTION

Large numbers of new foods and food components have been launched on the food and pharmaceutical market, attracting the attention and interest of consumers due to their acclaimed medical or health benefits. Products defined as probiotic or symbiotic, nutraceutical, functional, are now well described from a clinical and scientific point of view and are recommended for the enhancement of human health, for protection against several diseases or for the supply of essential metabolites with dietary and therapeutic characteristics [1,2]. Some of these foods contain probioctics microorganisms (bacteria and yeasts).

Presently, the principal yeasts used as probiotic belong to the genus *Saccharomyces* (S.) [3]. In particular, the strain *S. boulardii* is the only one commercialized with this purpose in human medicine [4]. *Saccharomyces boulardii* was first isolated from fruit in Indochina in the 1950s and was initially identified as a separate species of the genus *Saccharomyces*. Nevertheless, *S. cerevisiae* and *S. boulardii* are reported in the scientific reports for their biotherapeutic activities in the prevention of several types of diarrhea and colitisin humans [5,6]. The promising activities of yeasts as well as their ability to survive during the passage through the human gastro-intestinal (GI) tract, tolerating exposures to low pH and to bile salts, have drawn attention to their possible use as probiotics [7] even if their use for humans is currently restricted [8]. Furthermore, few studies have specifically focused on selecting or studying new probiotic yeast strains [4,8,9]. Given also the nutritive value of whole yeast cells of *S. cerevisiae* as sources of proteins, B complex vitamins and essential minerals [10,11] studies on the screening and selection of new probiotic yeast strains from foods in which the occurrence of yeast is already demonstrated could be considered an interesting research field. Such microorganism can be found in fermented foods. Palm wine generally refers to a group of alcoholic beverages obtained by fermentation from the saps of palm trees [12-14]. It is a whitish liquid produced by natural fermentation of the sap of palm oil tree, coco tree and Raphia [15,16]. Upon fermentation by the natural microbial flora, the sugar level decreases rapidly as it is converted to alcohol and other products [17]. Presence of micro-organisms such as yeasts, and bacteria has been reported [18-21]; However, yeasts and bacteria (LAB) are believed to play the most important role. The predominant yeast reported is *Saccharomyces cerevisiae*, but other yeasts are also commonly detected [14,22].

Studies have been performed on various aspects of palm trees in Cameroon but till date, to the

best of our knowledge, no study has addressed the physicochemical characteristics and microbiology of the wine prepared from the sap of the raffia palm trees present in the country. In palm wine fermentation in general, most published information have focused on the role of yeasts, but full identification to species level is very rare (non-existent for palm raffia from Menoua, West Cameroon region) and seem to be nonexistent. Certain yeasts are effective in the treatment of acute diarrhea in children [23,24] and release polyamines which help in repairing mucous membranes. These polyamines increase the activity of short chain fatty acids (SCFA) and disaccharide enzymes (lactase, maltase, sucrase). The beneficial properties of *Saccharomyces spp*. strains are well documented [25,26]. In addition to their nutritive value (e.g. provision of vitamins of the B group), probiotic yeasts are generally resistant to gastrointestinal passage and are resistant to most antibiotics. Yeast preparations have also been successfully applied, in combination with antibiotics, to treat *Clostridium* difficile-related diarrhea commonly known as antibiotic associated diarrhea. Probiotic yeasts may also help to re-establish a normal gut function, after long term antibiotic therapy [26,27]. *In vivo* studies showed that the administrations of probiotics and/or prebiotics are effective in improving lipid profiles, including the reduction of serum/plasma total cholesterol, LDL-cholesterol and triglycerides or increment of HDL-cholesterol. However, other past studies have also shown that probiotics and prebiotics had insignificant effects on lipid profiles. Several mechanisms have been hypothesized, which include: enzymatic deconjugation of bile acids by bile salts hydrolase of probiotics [28], assimilation of cholesterol by probiotics [29] co-precipitation of cholesterol into the cellular membranes of probiotics during growth [30] and production of short chain fatty acids upon fermentation by probiotics [31].

Traditionally, identification and characterization of yeast species has been based on morphological traits and their physiological capabilities [32,33]. This conventional methodology requires the evaluation of some 60 to 90 tests, resulting in a complex, laborious, and time-consuming process. In recent years, rapid kit identification methods have been developed to overcome the complexity of traditional methods [34-36]. One of these methods, the API 20 C AUX system (Biomèrieux, Lyon, France), has been widely used and consists of 19 assimilation tests. A recently developed kit, the Rap ID Yeast Plus system (Remel, Lenexa, Kans.), enables identification in only 4 hours. This method, although based on physiological properties, does not require yeast growth for biochemical test evaluation and dramatically reduces identification time. Unfortunately, all yeast identification kits were originally designed for clinical diagnosis and their application is generally restricted to few yeast species.

Cholesterol acts as a risk factor in different diseases such as cardiovascular diseases, colon cancer and hypercholesterolemia [37]. Results in recently published works indicate that the reduction of excessive levels of cholesterol in blood decreases the risks of these diseases but these have not been proven using raffia wine. Some natural microorganisms in human intestine are beneficial in terms of lowering serum cholesterol [38]. There is growing interest in the use of nutraceutical products which include probiotics and prebiotics and related metabolites with cholesterol lowering properties to prevent cardiovascular diseases [39]. Modifications of the intestinal flora have been shown to be beneficial on lipid metabolism in mice [40] and rats [41]. In contrast studies in humans [42] indicate that the role of fermented milk products as by hypocholesterolaemic agents were documented in the recent clinical studies [43] but this has not been proven using raffia wine. The cholesterol lowering effects of probiotics was found to be highly strain specific as different strains exhibit different levels of cholesterol lowering activity [44,45]. The assurance that probiotic yeast can cure obesity by reducing the cholesterol level will help cholesterolemic patient to switch from drugs with numerous side effects to natural drugs which have no side effect. These natural drugs are less expensive as compared to the synthesized drugs. This study aimed to isolate and partially identify probiotics yeast from raffia wine and to evaluate *in vivo* and *in vitro* their cholesterol lowering properties in rats.

2. MATERIALS AND METHODS

2.1 Sample Collection and Media Preparation

Samples of raffia palm wine that were used in this study were collected from Foto and Foreke areas of Menoua subdivision of West Cameroon and collected from 6 different palm wine tappers. The samples were collected in sterile sampling tubes and transported in ice cool boxes (4℃) to

the Life Science Laboratory of the Faculty of Science, University Buea, Cameroon. All media were prepared following the manufacturer's instructions. The media used were Sabouraud Dextrose Agar (Oxide) for isolation of yeasts, Aseptic techniques were observed throughout the media preparation process. A 1:10 dilution of each sample was made prior to culturing. This was done by diluting 1 ml of the sample with 9 ml of peptone water. Further, ten-fold serial dilutions ranging from 10^{-1} to 10^{-5} were prepared. The 10^{-5} diluted samples were used for culture on Sabouraud Dextrose Agar.

2.2 Yeast Isolation and Morphological Characterization

From 6 Samples of fresh raffia wine, 1 ml of wine was mixed with sterilized 9 ml peptone water then a serial of dilution was made until 10^{-5}, 1 ml of the last dilution was cultured by pour plate method using Sabouraud Dextrose Agar pH 5.5 [46]. Morphological appearance and microscopic examination of the colonies were done after staining them with methylene blue. The purity of the isolates was monitored with a light microscope. The morphology of the yeast genus was determined by microscopic observation after fixing and staining the cells with cotton blue dye. Direct observation ensured morphology glaze isolated cells, round or oval shapes and sometimes having budding while controlling their homogeneity by identifying potential contaminants. Yeast isolates were cultured at 25℃ for 48 h in Yeast-Peptone-Dextrose (YPD) broth Containing 0.5% (w/v) yeast extract, 1% Peptone and 2% glucose. YPD Agar (0.5%(w/v) yeast extract, 1% peptone, 0.5% glucose and 1.8% Agar) was used to maintain the cultures. Prior to the experiment, the cultures were activated for 72 h at 25℃ and subcultured at least three times.

2.3 Study of Acid, Bile Tolerance and Cholesterol Assimilation in Culture Media (*In-vitro*)

2.3.1 Tolerance to acidity

Yeast cultures were activated in YPD and to: incubated at 37ºC for 48 hours, then the biomass was separated by centrifugation (at 6000 x g/10 minutes). Sterile phosphate buffered saline PBS was added to the cells and mixed by vortex mixer. Tubes were inoculated with (10^6 CFU/ml) of yeast cells in which the pH was adjusted (pH1, 2 and 3) using HCl 0.1 N. The tubes were incubated at 37ºC and OD was read at 560nm. The formula described by Park et al. [47] and Brashears et al. [48] was used to calculate the percentage of survival in the culture:

> % of survival = OD of the culture after (X) hour pH Y - OD of the culture at 0 hour pH Y x 100 / OD of the culture at (X) hour pH 7 - OD of the culture at 0 hours pH 7
>
> X = 6 or 24; Y = 2 for a same wavelength and the same isolate.

2.3.2 Bile salt tolerance

Yeast cultures were activated by two transfers in liquid Yeast Peptone Dextrose (YPD) and inoculated at 37ºC for 48 hr and centrifuged at 3000 rpm for 10 minutes, then sterile phosphate buffered saline was added to the cells and mixed by vortex mixer, then the tubes contain YPD medium supplemented with different concentrations of bile salt (0.2 and 0.4) and inoculated with (10^6 CFU/ml) of yeast cells. The growth of the yeast cells was estimated after 6 hours and 24 hours of incubation at 37ºC by measuring absorbance at 560 nm. The percentage of survival was calculated using the modified formula described by Park et al. [47] and Brashears et al. [48].

2.3.3 Cholesterol assimilation from culture media

Freshly prepared Broth was poured into 14 screw tubes in duplicate. Half part of the tubes was supplemented with 0.2% bile salts and the rest with 0.4% bile salts following by the addition of 1% cholesterol in all the tubes. They were inoculated with 1% of respective cultures (1R27, 1R28, 2R29, 3W29, 2R30, 1W29 and 1R31) while under the same conditions four other tubes were prepared free of isolates and used as control. They were incubated at 37℃ for 24 h. After incubation, cultures were centrifuged and unutilized cholesterol was estimated in the supernatant by measuring the optical density at 540 nm and compared to the control. Isolates (1R27, 2R29, 1W29, 3W29) having the highest in vitro reduction of cholesterol on the media were selected for API identification.

2.3.4 Strain identification using the API 20C AUX

The identification of the 4 yeast isolates was done by biochemical characterization using the

API 20 C AUX kit (BioMerieux, France). API® 20 C AUX Molten (50℃) API basal medium ampoules were inoculated with yeast colonies, and the suspension was standardized to a density below 1+ (lines can be clearly distinguished) on a Wickerham card. Each cupule was inoculated, and the trays were aerobic incubated for 72 h at 30℃. Cupules showing turbidity significantly heavier than that of the negative control cupule (0 cupule) were considered positive. Identification was made by generating a microcode and using the API® 20 C AUX Analytical Profile Index or the Voice Response System (for profiles not found in the index). Morphology on cornmeal was also evaluated as determined by the manufacturer. The biochemical profile obtained for the yeasts strains was analyzed using the API identification software database (API LAB PLUS), Version 5.

2.4 *In vivo* Evaluation of the Effect of the Probiotics Strain on the Lipid Profile

Among the 4 yeasts isolates previously identified only two (1R27 and 3W29) with the best *in vitro* cholesterol assimilation were selected for the *in vivo* assay.

2.4.1 Animal feeding and experimental design

24 wistar albino rats (60 – 90 g) of about 21 days obtained from the animal house of the Department of Zoology, University of Buea were separated into 4 groups of 6 rats each (3 males, 3 females), the negative control group (fed with basal diet + oral gavage of deionized water) termed A, the positive control group (received hyperlipidemic diet (about 85% basal diet, 1% cholesterol, 10% lard (pig fat), W/W + oral gavage of deionized water) termed B, and 2 test groups (fed with hyperlipidemic diet and received yeasts strain (1R27 *Sacharomyces cerevisiae* and 3W29 *Candida famata*) termed group C and D respectively. They were housed under standard conditions with a 12 h light and 12 h dark cycle. Temperature was maintained at 23 ± 2℃ and relative humidity at approximately 50%. The rats were acclimatized for 7 days prior to use. The animals were housed in metabolic cages and given standard diet (commercial pellet formula adapted from Malathi et al. [49] formula with slight modifications) and water ad libitum throughout the study. The experiment was carried out for four weeks at oral gavages dose level 10^8-10^{10} CFU/ ml at a volume of 1.0 ml/kg body weight/day for each dose. The amount of food consumed and animal's weight were recorded daily. The composition of the basal diet is shown on the Table 1.

2.4.2 Rat blood collection and biochemical assay

After dosing (30 days), the rats were allowed to fast overnight (12 hours) and on the 31st day, anaesthetized using chloroform and sacrificed. Blood was collected by cardiac puncture into eppendorf tubes and the whole blood was centrifuged for 10 minutes at 4000 rpm to obtained serum. The sera were kept at -20℃ for the analysis of the lipid profile.

Serum lipid profile, triglycerides (TG) were determined with the use of commercial kits. Total cholesterol (TC) was analyzed enzymatically with CHOD/PAP Method [50-52] and HDLc was also estimated with precipitation using commercial kits [53]. Serum LDLc was determined according to the Friedewald formula with use of HDLc and total cholesterol values [54]. VLDL-cholesterol (VLDL-c) was calculated by dividing triglyceride concentration by five. HDL/LDL ratio was calculated. Albumin, serum activities of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were measured using a commercially available kit via the methods described respectively by Tietz et al. [55], Doumas et al. [56] and Murray-Kaplan et al. [57]. An ELISA plate reader (Labsystems, MultiskanEX, Finland) was used to read the absorbance. All the kits used were manufactured by CHRONOLAB SYSTEMS in Barcelona, Spain and were used according to the manufacturer's instructions.

After the last day of administering isolates, the rats were fasted overnight for twelve hours then sacrificed. They were dissected and their blood samples collected in eppendorf tubes aseptically together with vital organs like the liver, kidneys, spleen and heart which were aseptically handled and put in an ice bath. Analysis was done 2 weeks later to determine the effect of the probiotic yeasts isolates on the rats.

2.5 Statistical Analysis

Data obtained were analyzed with the aid of Microsoft Excel 2010 software for windows. Data are presented as the mean ± standard deviation and P value < 0.05 was considered to be significant. Comparisons were made by use of the Bonferroni tests performed using Graphpad Insat 2000 software.

Table 1. Composition of diet and rats groups

Ingredients	Rat group and types of diets		
	A (Control) Basal diet	B (Positive control) Hyperlipidemic diet	C or D (Test groups) Bacteria isolates
Soy meal	20	20	20
Corn Starch	60	56	56
Sugar	10	3	3
soybean oil	5	5	5
Choline and Methionine	0.5	0.5	0.5
Mineral mixture	3.5	3.5	3.5
Vitamin mixture	1	1	1
Cholesterol	0	1	1
Lard	0	10	10
Total:	100	100	100
Gavage substance	Distilled H_2O	Distilled H_2O	*Saccharomyces cerevisae* or *candidata famata*

3. RESULTS AND DISCUSSION

3.1 Isolation and Selection of Yeasts Isolates

From 6 samples of palm wine, 28 had morphological appearance of yeast. Among them, acid tolerance test permitted the selection of 12 isolates based on their more than 50% resistance to acid medium; while bile tolerance test permitted the selection of 7. Four out of the selected 7 were retained for their high *in-vitro* cholesterol assimilation on culture media and were further identified as species level as *Saccharomyces cerevisiae, Candida famata, Cryptococcus neoformans* and *Candida famata*.

3.2 Acid Tolerance of Cultures (Effects of Low pH)

28 yeast isolates were first characterized base on their adaptation to acid conditions (pH 1, 2 and 3) close to the human gastro intestinal tract (Table 2). Among them 12 survived. The survival of the 12 isolates in medium of pH 3 was high after 6 hours of incubation at 37℃ compared to the survival rate after 24 hours (Table 2). Decrease in pH to 2 and later round to 1 progressively led to further reduction in cell viability and the survival rate. The wine isolate showed higher sensitivity to pH 1 and 2 and their survival ranged from 96.56 to 54.05% at pH 3 after 6 hours of incubation against 67.92 to 4.58% after 24 hours. In this study, the abilities of yeast strains to endure low pH (1, 2, and 3) were investigated. All the 12 yeast isolates presented more than 50% survival at pH 3.0 after 6 hours of incubation while after 24 hours only isolate 1R27 recorded high % survival (67.92%). However, at pH 3 isolates 1R27, 1R28, 2R29, 3W29, 2R30, 1W29 and 1R31 displayed very high viability; the yeast isolates displayed variability in surviving in simulated gastric juice. These isolates would then exhibit stronger resistance to gastric juice. It has been reported that the abilities of probiotic to survive transit through the gastrointestinal tract are variable and strain dependent [58]. This conclusion is in accordance with the previous report of Psomas et al. [59] on infant feces. Most definitions of probiotics emphasize that the microorganism should be viable and reach their site of action alive [60]. The primary barrier in the stomach is the gastric acid of inhibitory action being related to low pH and enzyme presence. All the tested yeasts isolated at pH 3 after 6 hours showed high tolerance to these conditions, however raffia wine isolates were more sensitive at pH lower than 3. It seems that the factor which could highly influence the growth of yeasts was pH. Normal values for human gastric pH are 1-3 fasting and up to 5 hours after a meal [61] and average stomach transit time is 2.5-4 hours [62]. We assumed stricter conditions in our investigation and examined yeasts survival during 6 and 24 hours of incubation in medium of very low pH. In these harsh conditions sufficient viable cells of all the tested strains could enter the small intestine indicating the possibility of their survival and proper activity in the human intestine.

3.3 Bile Salts Tolerance Tests: Effects of Bile Salts on Viability

Among the 12 isolated selected for acid resistance test and bile tolerance, the 7 best at pH3 which were able to have high percentage survival in Oxgall medium are presented in Table 3. According to the suggestion of FAO/WHO, it is

mandatory to perform a preliminary *in vitro* assessment before assessing the probiotic properties of bacterial strains [63] such as the capacity for bile tolerance to ensure survival during passage through the gastrointestinal tract [64]. Regarding the ability of yeasts to withstand bile salts, all yeast isolates were incubated in bile salts solutions of 0.2% and 0.4% for 6 and 24 hours. As shown in Table 3, the growth of most isolates (1R27, 2R29, 1W29 and 3W29) at 0.2% Oxgall and 1R27, 2R29 and 1W29 at 0.4% Oxgall after 6 hours of incubation was not affected by the addition of bile salt as the control; While after 24 hours, only 1R27 at 0.2 and 0.4% Oxgall were able to grow for more than 100% indicating that the medium and condition were favorable or propitious to their live then were no inhibited by the bile salt solutions. But in general four isolates 1R279, 2R29, 3W29 and 1W29, after 6 or 24 hours incubation showed good bile resistance, even at a concentration of 0.4%. In the presence of 0.2% and 0.4% bile salts, 3 isolates showed percentage survival less than 97%. Besides tolerance to acid conditions, all the tested yeasts demonstrated the ability to withstand 0.2 and 0.4% Oxgall. Similar results have been reported previously for *S. cerevisiae* strains isolated from infant feces, feta cheese and beverages [8,65]. Bile tolerance is important for allowing a microorganism to survive in the intestinal tract [66]. Growth at 37℃ seems to be a variable characteristic of *S. cerevisiae* [32]. In this study the raffia wine isolate were able to grow at this temperature. This finding is consistent with the conclusion of the importance of yeast origins for probiotic properties [67]. However, irrespective of strain origins, all the tested yeasts may survive passage throughout the upper gastrointestinal tract and be viable at their sites of action in the gut environment. The results of the present study suggest that all tested yeasts and particularly isolates 1R27, 2R29, 3W29 and 1W29 may survive in the human gastrointestinal tract and thus create the possibility of proper activity in the human body.

3.4 *In-vitro* Cholesterol Assimilation on Culture Media

The 7 isolates were submitted to cholesterol assimilation on culture medium and 4 showed best results summarized in Table 4.

Table 2. *In vitro* survival of selected yeasts isolates in simulated gastric juice conditions

Isolates code	Acid tolerance					
	% Survival after 6 hours of culture			% Survival after 24 hours of culture		
	pH 1	pH 2	pH 3	pH 1	pH 2	pH 3
1R27	15.69	18.77	96.56	14.37	17.18	67.79
2R27	13.10	29.70	58.5	3.36	8.41	18.77
1R28	31.89	45.77	92.66	24.35	35.42	43.54
3R28	4.98	8.04	56.85	4.08	4.59	4.78
2R29	42.43	51.66	94.7	3.00	7.22	5.45
3W29	8.02	27.80	70.58	2.13	4.90	5.88
1R26	10.29	13.9	60.99	8.7	13.26	32.67
2R30	5.69	34.52	54.52	0.00	17.3	27.6
1W29	34.39	78.1	94.52	4.36	5.83	17.70
8R26	34.39	79.78	85.1	0.00	2.76	18.38
1R31	5.98	40.86	78.47	3.64	36.39	47.82
3530	18.92	40.50	54.05	4.58	4.58	8.41

Table 3. Yeast growth in the presence of different bile salts concentration

Isolates code	Bile salts tolerance tests			
	% Survival after 6 hours of culture		% Survival after 24 hours of culture	
	Bile 0.2%	Bile 0.4%	Bile 0.2%	Bile 0.4%
1R27	182.93	146.07	142.18	135.02
1R28	58.00	65.00	48.00	52
2R29	135.13	134.23	74.18	84.13
3W29	106.15	86.52	55.68	66.27
2R30	84.41	62.01	59.58	52.95
1W29	108.96	107.34	65.95	78.97
1R31	86.25	97.00	53.20	63.4

High levels of serum cholesterol have been associated with the risk of coronary heart disease, and the use of probiotic bacteria to reduce serum cholesterol levels has attracted much attention. The abilities of 4 selected yeasts isolates to assimilate cholesterol in the presence of bile salts are shown in the Table 4. All yeast isolates could assimilate cholesterol from the media containing bile salt and cholesterol after 24 hours growth at 37℃. Four yeast isolates (1R27, 2R29, 3W29 and 1W29) had cholesterol assimilation abilities ranging from 46.04% to 61.72% at 0.2% bile salts while at 0.4% bile salts, all the for assimilate more than 505 cholesterol. Among the 4 yeast isolates, 1R27 and 3W29 had the highest cholesterol assimilation ability then displayed the best cholesterol assimilation ability yeasts have previously been evaluated as potential probiotics for assimilating cholesterol over the past few years [59,68]. Several mechanisms have been hypothesized to explain the cholesterol reducing properties, which include enzymatic deconjugation of bile acids by bile-salt hydrolase of probiotics [28] assimilation of cholesterol by probiotics [69], co-precipitation of cholesterol with deconjugated bile [70], cholesterol binding to cell walls of probiotics [71], incorporation of cholesterol into the cellular membranes of probiotics during growth [30], conversion of cholesterol into coprostanol [72] and production of short-chain fatty acids upon fermentation by probiotics in the presence of prebiotics [73]. Hypercholesterolemia is a major risk factor for coronary artery disease and probiotics can then be suggested as tools to manage elevated cholesterol levels.

3.5 Strain Identification Using the API 20 C

After in-vitro test of cholesterol assimilation on culture media, 4 isolates of yeast (1R27, 2R29, 3W29, and 1W29) were selected for their high in-vitro cholesterol lowering effect for identification using API 20 C AUX; results obtained are summarized in Table 5. D-Glucose, D-Xylose, D Galactose, Methyl-αD-Glucppyraoside, D-Maltose, D-Saccharose, D-Raffinose, were utilized by *all the strains while no one of* then showed utilization to Inostol. 1R27 showed higher utilization rates with D-Glucose, D-Xylose, D Galactose, Methyl-αD-Glucppyraoside, D-Maltose, Saccharose, D-Raffinose. In addition to sugar utilised by 1R27, 3w29, also utilize 2Ketoglutarate, D Sorbitol, N-Acetyl glucosamine, D-cellobiose, D-Trehalose, D-Lactose, D-Melizitose. Comparison with the API database (https://apiweb. Biome rieux.com) [74] revealed 94.9%, 95.3%, 51.5% and 92.2%, homology of 1R27, 1W29, 2R29 and 3W29 with *Saccharomyces cerevisiae, Candida famata, Cryptococcus neoformans* and *Candida famata* respectively. Chandrasekhar et al. [75] Nwachukwu et al. [76] also reported the presence of *saccharomysiae* and *candida* as microorganism found in palm wine; these same strains was also isolated by Abdel et al. [77] during isolation and identification of yeasts from the different stages of Hulu-mur Fermentation. Based on the best *in vitro* cholesterol assimilation of 1R27 and 3W29, they can therefore be used to check for their cholesterol lowering effect in *in-vivo* studies.

Table 4. Cholesterol assimilation of selected yeasts isolates at different bile salts concentration

Isolates code	% cholesterol assimilation	
	0.2% Bile salts	0.4% Bile salts
1R27	61.72	68.76
2R29	46.04	56.78
3W29	59.2	62.59
1W29	56.04	51.08

3.6 Rats Bioassay

3.6.1 Feed consumption of rats

Even if in each group the quantity of feed consumed increase with time , in general, there was no significant difference observed in point of rat's food consumption between the experimental groups receiving only high fat diet and test group receiving hyperlipidemic diet and yeast strains during the four weeks of feeding period (P>0.05) (Table 6). Compare to the control (group A) test groups and hyperlipidemic group showed significant increase of food consumed probably due to the high fat contain of that diet since oil increase palatability of food. It had been reported that fat plays a unique role in the human diet. In addition to being the most concentrated source of dietary energy, fat contributes to the texture, flavor, and aroma of a wide variety of foods. In general, the most palatable foods are those that are both energy-dense and high in fat content [78]. The taste, smell, mouthfeel, and hedonic properties of fat all contribute to the popular concept of fat "taste" [78-81].

Table 5. API test results after 48 hours of incubation of selected yeast

Test number	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Identification API
Strain code		D-Glucose	Glycerol	2Ketoglutarate	L-Arabinose	D-Xylose	Adonitol	Xylitol	D Galactose	Inostol	D Sorbitol	Methyl-αD-Glucpyraoside	N-Acetyl glucosamine	D-cellobiose	D-Lactose	D-Maltose	D-Saccharose	D-Trehalose	D-Melizitose	D-Raffinose	
1R27	-	+	-	-	-	+	-	-	+	-	-	+	-	-	-	+	+	-	-	+	*Sacchoromyces cerevisiae*
3w29	-	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	*Candida famata*
2R29	-	+	-	+	-	+	+	-	+	-	+	+	+	-	+	+	+	+	+	+	*Crytococcus neoformans*
1w29	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	*Candida famata*

+, positive reaction; -, negative reaction

Table 6. Weekly food consumption of rats

Weeks	Rats group and weekly food consumed (g)			
	A (Control)	B (Positive control)	C (Test) Isolate 1R27	D (Test) Isolate 3W29
1	14.04±1.45[a]	15.4±0.07[b]	16.6 ± 0.99[b]	17.04±2.47[b]
2	14.55±2.53[a]	18.33±0.17[b]	19.7 ± 0.39[b]	18.84±0.04[b]
3	14.52±0.79[a]	20.91±0.19[b]	21.2 ± 1.27[b]	19.04±2.55[b]
4	14.00 ±0.79[a]	21.34±0.79[b]	22.65 ± 1.69[b]	21.014±0.49[b]

Values with different superscript on a line are significantly different (P < 0.05)

Table 7. Organs weight and weekly weight gained by the rats

Weeks	Body weight gain (g)			
	A (control)	B (positive control)	C (Test) Isolate 1R27	D (Test) Isolate 3W29
1	15.30±1.14[a]	16.74±1.01[a]	14.18±2.51[a]	15.09±0.03[a]
2	18.31±0.01[a]	24.07±0.01[b]	15.67±1.46[a]	16.06±0.12[a]
3	14.91±0.01[a]	21.68±0.01[b]	17.41±1.41[a]	18.45±0.09[a]
4	16.55±1.06[a]	22.87±1.00[b]	19.96±0.18[a]	19.34±0.88[a]
Organs	**Organs weight (g)**			
Liver	6.90±1.00[a]	8.20±0.01[b]	7.20±1.24[a]	6.70±1.40[a]
Kidneys	0.70±0.07[a]	1.11±0.01[a]	1.00±0.08[a]	0.80±0.00[a]
Spleen	0.40±0.01[a]	0.30±0.01[a]	0.45±0.17[a]	0.44±0.00[a]

**Values with different superscript on a line are significantly different (P < 0.05)*

3.6.2 Weight gain and organs weight

There was no significant difference observed in point of rat's weight between the experimental groups during the first week of feeding period (*P*>0.05) (Table 7). While the highest weight gain was observed in hyperlipidemic group (group B), the lowest weight gain was observed in control (groupe A) and test group (groupe C and D) indicating that despite high food consume with the high fat diet yeast strain help to avoid weight gain. Cholesterol and fat supplementation to basal diet provoke increase in weigh but feeding with yeast supplementation provides decreasing on weight. Endocannabinoids released after palatable food ingestion, such as food containing fat and sucrose, will promote hunger and energy storage leading to weigh increase. The following hormones have been found to regulate the appetite for fat. Galanin [81], agouti-related peptide (AgRP) [79], and ghrelin [80] stimulate fat intake and then can lead to hyperlipidemia, obesity and fat storage. Yeast strain here might prevent cholesterol, fat storage or fat absorption. They can improve digestive health, reduce inflammation, improve cardiovascular risk factors and even help fight depression and anxiety. There is also a lot of evidence that obesity is linked to inflammation in the brain. By improving gut health, probiotics may reduce systemic inflammation and protect against obesity and other diseases [82,83]. Probiotic may help release the satiety (appetite-reducing) hormone GLP-1. Increased levels of this hormone may help you burn calories and fat [84] Probiotics may increase levels of the protein ANGPTL4. This may lead to decreased fat storage [85].

It is thought that certain probiotics may inhibit the absorption of dietary fat, increasing the amount of fat excreted with feces [86]. There are many different microorganisms in the gut, mostly bacteria. Several lines of evidence suggest that these gut bacteria can have powerful effects on body weight. Probiotics might be able to help improve blood sugar and body weight, according to a new study showing that the probiotic yeast *Saccharomyces* reversed diabetes and obesity by improving gastrointestinal health and reducing inflammation.

Organs weight of the different groups of rat after feeding period is summarized in Table 7. Results are showing that except group B (hyperlipidemic or hypercholesteromic group) which recorded significant increase in liver weight. Rats organs were not negatively affected with the dose of yeast administrated.

Hatice et al. [87] reported in a similar study on rats fed on a high cholesterol enriched diet that increase in liver weight can lead to steatosis and caseation necrosis. Kidneys and spleen were normal as a control. There was no observed any significant decrease in organs weight on Group C and D which was fed with yeast.

3.6.3 Serum biochemical analysis and lipidemia

As seen in Table 8, there was no significant difference between the Group A, C and D in terms of the levels of total cholesterol, triglyceride LDL and HDL cholesterol (P>0.05). Yeast administration had effect on level of total cholesterol; HDL cholesterol and triglyceride compare to group B (hyperlipidemic group free of yeast strains). But it led reduced LDL cholesterol of test group as in control compare to hypercholesterolemic group (B). In general, *in vitro* high assimilation rate was determined in the medium and could not be observed *in vivo* because metabolism synthesizes cholesterol when needed and our bodydon't need exogenous cholesterol. These results could be considered as promising, because 1% reduction in cholesterol can reduce the risk of cardiovascular diseases for 2-3% [88]. In this respect, the selection of probiotic strains which have high percentage of cholesterol assimilation rates is important. Nowadays, probiotic yeasts can be delivered either in fermented foods or as cultures administered orally. Several yeasts species have been used in many probiotic preparations [89]. Several studies have shown the ability of probiotic microorganisms to lower cholesterol levels in vivo. Bertazzoni et al. [90] found lower serum cholesterol (3.73%-13.49%) and triglyceride (9.30%-40.23%) ratios and stated an increase on HDL cholesterol level in fats fed on fermented milk containing Lactobacillus casei compared to control group after 10 days. Nguyen et al. [91] Beena and Prasad [92] found lower serum cholesterol in rats fed on yoghurt containing probiotics after 30 days. The mechanism(s) responsible for the cholesterol-lowering effect of probiotics remains unclear, but it has been suggested that the effect could be obtained through retarded cholesterol synthesis and increased degradation of cholesterol [93]. Diets supplemented with probiotics can also significantly reduce plasma triglycerides in broilers. In a study, broilers fed with addition of 0.5% *Saccharomyces cerevisiae* triglyceride and total cholesterol levels decreased at the rate of 22.67 and 9.95% respectively after 3 weeks [94]. Cholesterol level was significantly lower in broilers supplemented with thermo-tolerant probiotic yeast at different levels compared to control group [93]. Oral administration of probiotics has been shown to significantly reduce cholesterol levels by as much as 22 to 33% [95]. Further, decrease in cholesterol content of eggs of laying hens and broilers diets containing yeasts was reported by Yalçın et al. [96] and Yıldız et al. [97]. Seyidoğlu and Galip [98] evaluate the effect of *Saccharomyces cerevisiae* on the serum biochemical parameters in rabbits, the diets with the yeast reduced serum HDL cholesterol and triglycerides on the 90th feeding day. Similar to our findings serum cholesterol slightly decreased by the yeast. In a study performed by Güven and Güven [99] on fermented kefir grains there was significant suppression in serum lipids on the rabbits fed with cholesterol supplemented diet.

The aminotransferases (transaminases) are sensitive indicators of liver cell injury and are most helpful in recognizing acute hepatocellular diseases such as hepatitis. The pattern of the aminotransferase elevation can be helpful diagnostically.

In this study, Table 8 shows the AST: ALT ratio 8.66 for normal rats, no consistent with De- Ritis et al. [100]. Yeast administration showed lower impact on transaminase (ASAT and ALAT) rate of test group (groupe C and D) compare to the control (A) but hypercholesterolemic or lipidemic diet cause increase of those enzymes but the presence of yeast on hyperlipidemic diet help reduce this enzyme rate as in negative control group. In our study, non-addition of yeast in hyperlipidemic group affects the amount of serum enzyme in rats. Blood tests used for initial assessment of liver disease include measuring levels of serum Alanine and Aspartate aminotransferases (ALT and AST), alkaline phosphatase, and others. The pattern of abnormalities generally points to hepatocellular versus cholestatic liver disease and helps to decide whether the disease is acute or chronic and whether cirrhosis and hepatic failure are present [101]. Serum enzyme levels fluctuate widely from normal to moderately abnormal, with values rarely into the high hundreds [102-104]. Marked elevation of aminotransferases in the appropriate clinical context indicates acute cell necrosis caused by viral infection, drugs, toxins, alcohol, or Ischemia [105].

Table 8. Serum lipid indices for the period of basal diet and basal diet plus yeast isolate consumption

Parameters	A (Control)	B (Positive control)	C (test group) 1R27	D (test group) 3W29
TC mg/dL	120±3.30[a]	200.49±10.83[b]	101.55±3.30[a]	102.08±3.30[a]
TG mg/dL	96.24±0.24[a]	277.820.59[c]	115.57±3.30[a]	115.86±3.30[a]
HDL-c mg/dL	60.83±0.06[a]	43.39±0.01[b]	66.67±3.30[a]	64.09±3.30[a]
LDL-c mg/dL	11.053±7.95[a]	101.54±5.00[b]	11.76±3.30[a]	14.81±3.30[a]
VLDL-c	19.248±2.13[a]	55.56±0.004[c]	23.11±3.30[a]	23.17±3.30[a]
HDLc/LDLc	5.50±0.0014[a]	0.42±0.68[b]	7.12±3.30[a]	4.32±3.30[a]
Albumin mg/dL	2.83±0.01[a]	5.05±1.01[b]	3.42±3.30[a]	3.16±3.30[a]
AST (U/L)/	131.47±78.01[a]	831.249±44.01[b]	130.43±3.30[a]	117.92±3.30[a]
ALT (U/L)	15.17±0.01[a]	176.17±10.01[b]	20.15±3.30[a]	20.73±3.30[a]
De Ritis ratio (AST /ALT)	8.66	4.72	6.47	5.69

TC: Total cholesterol, TG: Triglyceride, HDL-c: High¬ Density Lipoprotein-cholesterol, VLDL-c: Very Low Density Lipoprotein-cholesterol;
LDL-c: Low Density Lipoprotein-cholesterol, ALT: alanine aminotransferase, AST: aspartate aminotransferase.
Data are presented as mean ± SD; All the administered dose were 1.0 ml/kg day
Values with different superscript on a same line are significantly different from control at P < 0.05.
A, B and C, are the different groups of rats fed with their respective diets

4. CONCLUSION

Probiotic yeast administration has beneficial effects by lowering total cholesterol, LDL and triglycerides. It was observed that the level of HDL increase following yeasts oral administration, reducing rate of triglyceride level was higher than many in vivo studies. It is therefore encouraged that additional efforts are placed on exploring the health beneficial effects of yeasts. In conclusion, with the procedure employed in this research we selected four yeast strains capable of surviving the low pH and also the simulating bile salts environment of the human intestine, which make them suitable as potential probiotic strains as new supplements in foods or pharmaceutical preparations. This research represents a preliminary study of probiotic yeast selection; to declare their effectiveness; these strains would require further investigations such as antagonistic activities against human entero-pathogens, adhesion capability to the intestinal mucosa cells, characterization at molecular level and specific clinical analyses concerning human health.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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