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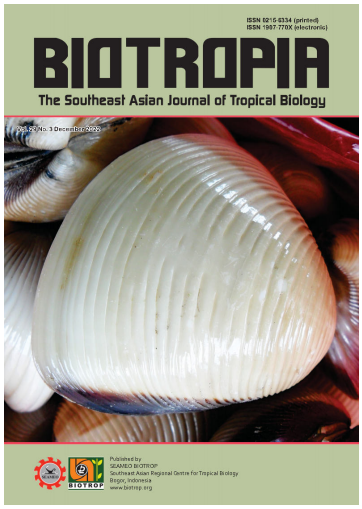

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Research Paper

The molecular approach reveals the relationship among Venus clams (Meretrix spp.) Community in Malaysia

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Dr. Mohd Hanafi Idris , Dr. Abu Hena Mustafa Kamal, Dr. Hadi Hamli, Dr. Amy Rajaee, Mr. Abdulla-Al-Asif

PDF

CADMIUM, NICKEL, AND LEAD CONCENTRATION OF MUNICIPAL DUMPSITE IN WESTER SAMAR,

Pearl Aphrodite Carnice

PDF

PHYSICAL AND CHEMICAL QUALITY OF CORN WITH DIFFERENT MOISTURE LEVELS SUPPLEMENTED WITH MOLD INHIBITOR

Different moisture levels of corn supplemented with mold inhibitor

CATOOTJIE LUSJE NALLE, Mr. Max A. J Supit, Mr Angga M. Akbar, Miss. Emiliana Langodai, Miss Anggriana Soo

PDF

ROLE OF MYCORRHIZA HELPER BACTERIA ON MYCORRHIZAL COLONIZATION AND NEMATODE PRATYLENCHUS COFFEAE INFECTION

Reginawanti Hindersah, Elena FL Lilipaly, Iis Nur Asyiah, Imam Mudakir, Rita Harni

PDF

Trees' Physiological Responses to Air Pollution in Taman Margasatwa Ragunan and Campus UI Depok

Diana Selvilia Hamid, Ratna Yuniati, Afiatry Putrika

PDF

The YEAST PROBIOTICS WITH POTENTIAL TO ASSIMILATE CHOLESTEROL IN VITRO

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Yan Ramona, Ni Luh Putu Ariwathi

PDF

MACROFUNGAL DIVERSITY IN DIFFERENT VEGETATION COMPOSITIONS IN TEGHARI COMMUNITY FOREST, KAILALI, WEST NEPAL

Hari Sharan Adhikari, Kausalya Joshi, Hari Prasad Aryal, Laxmi Joshi Shrestha

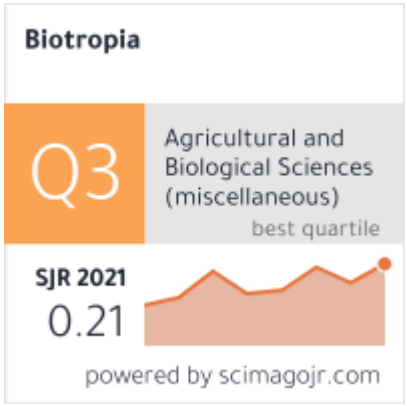
PDF

Information

For Readers

For Authors

For Librarians



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YEAST PROBIOTICS WITH POTENTIAL TO ASSIMILATE CHOLESTEROL *IN- VITRO*

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ABSTRACT

In the last two decades the use of yeasts as new probiotics has increased significantly. Therefore, our current research was focused on the investigation of yeasts for novel probiotic development in Bali. The main objectives of this research were to isolate and characterize yeasts isolated from *ragi tape* (dried mix cultures of microorganisms normally used in the fermentation of rice or cassava in Indonesia) and *tape ketan* (fermented sticky rice) for possible use as yeast-based novel probiotics, with capability to assimilate cholesterol *in vitro*. In this study, the potential yeast isolates were evaluated for survival at low pH conditions (pH 2, 3, or 4) and in high levels of sodium deoxicholic (NaDC), at concentrations of 0.2, 0.4, or 0.6 mM. In addition, the yeast isolates were also evaluated for their ability to assimilate cholesterol *in vitro* and to elucidate biotransformation of cholic acid into deoxycholic acid. This study led to 10 isolates that were resistant to pH levels of 2, 3, or 4 and to NaDC at concentration of higher than 0.4 mM. Most of those isolates were also found to assimilate cholesterol *in vitro* at the rate of between 18% and 76% in 24 hours incubation. In the biotransformation test, none of those isolates transformed cholic acid into deoxycholic acid, indicating that they are safe and have potential to be developed into novel probiotics, either for human or cattle.

Keywords: Bali, cattle, cholesterol assimilation, probiotics, yeast

INTRODUCTION

Intestinal probiotic has been defined as beneficial microbes residing along the intestinal tract of human or animals that provide beneficial effect to their host's intestinal health (Stasiak-Różańska *et al.* 2021). These exclude commensal microbes residing in the intestine that do not provide health benefit to their host (Lorenzo & Lucio 2012). Such commensals can be claimed as probiotics only if their health benefit can be demonstrated to their hosts. Probiotics appear for the first time in the intestinal tract of human or animals either during delivery of babies through reproductive tract of their mothers or at the first time of lactation (Quin *et al.* 2018). Depending on the types of foods in the infant period of their growth and development, alteration of microbial composition in their intestine occurs (Zhang *et al.* 2018). The role of prebiotics and fibers in their food has also been extensively reviewed by Holscher (2017) to understand the effect of prebiotics and fibers to the composition of human intestinal tract. According to Zommiti *et al.* (2020) probiotics applied as a single or mixed culture at the rate of 10^9 cells per day can improve the health of animal or human digestive system by maintaining the natural balance of the microbiota in the host's digestive tract. Besides, they can suppress or even exclude pathogenic microbes entering intestinal tract through mouth (Markowiak & Sli˙zewska 2017). The mechanisms of pathogenic growth suppression by probiotics *in-vitro* or *in-vivo* have been extensively reviewed by Maldano-Galdeano *et al.* (2019).

Probiotics are normally developed from nonpathogenic microorganisms that provide positive effects on the physiology of the intestine, if they are regularly consumed at sufficient density (Maldano-Galdeano *et al.* 2019). Many of them play important roles in the elimination of toxic compounds in the intestinal track (Petrova *et al.* 2022) resulting in an improved degree of host's digestive tract health.

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Many probiotic species also provide functional effects, such as preventing diarrhea (McFarland 2010), reducing cholesterol content in the blood (Nocianitri *et al.* 2017), or inducing the immune system of their hosts (Maldano-Galdeano *et al.* 2019). Besides these benefits, the role of probiotics as possible anti-cancer agents has been reviewed by Bhuvan and Saroj (2018) and Sankarapandian *et al.* (2022).

Until recently, commercial probiotics have been dominated by Lactic Acid Bacteria (LAB) isolated from healthy infants, aged under 3 months old (Ramona *et al.* 2015). These LABs have been recognized to be nontoxic, although some of them have been reported to produce antimicrobial compounds, such as bacteriocin (Gonzales-Perez *et al.* 2018). This ability enables the LABs to control the balance of normal microbiota in the intestinal tract of human or animals, so that they can prevent pathogenic microbes from infecting the host's intestinal tract.

Besides LAB, yeasts have recently been investigated for novel probiotic development, although research on yeast, such as *Saccharomyces* spp., is not as intensive as the study on LAB. Before being developed as potential and novel probiotics, some important specific characteristics of the yeasts, such as pathogenic properties, tolerance to upper part of intestinal tract conditions (e.g. low pH and high concentration of deoxicholic acid), and ability to convert cholic acid into deoxicholic acids need to be elucidated. In addition, the probiotic candidates should be nontoxic and provide beneficial effects, such as ability to reduce blood cholesterol (Nocianitri *et al.* 2017) content and improve immune system in their hosts (especially human) so that they can be consumed in a long period of time to derive benefits (Castellano *et al.* 2017).

Based on the above rational, yeasts (*Saccharomyces* spp.) were isolated from *ragi* (dried mix cultures of microorganisms normally used in the fermentation of rice or cassava in Indonesia) and *tape ketan* (fermented sticky rice), with the main objective to investigate some important characteristics (including their ability to assimilate cholesterol) of these isolates before being developed as novel and potential probiotics, in our present study.

MATERIALS AND METHOD

Isolation of Yeasts (*Saccharomyces* spp.) from *Ragi* and *Tape Ketan*

The yeasts, *Saccharomyces* spp., were isolated from *ragi* and *tape ketan* obtained from traditional markets around Denpasar City, Bali, Indonesia. The samples which had been processed through dilution and spread plate methods as specified in Ramona *et al.* (2015) were applied in the isolation and purification of yeasts with probiotic potential. Sample size of 1 g of ground *ragi* or *tape ketan* was diluted in 9 mL of saline solution to obtain dilution rate of 10^{-1}. This was further diluted to 10^{-5} in the saline solution. Subsequently, 100 µL of suspension was taken from test tubes with dilution rates of 10^{-4} and 10^{-5} and was spread on plates containing Potato Dextrose Agar (PDA), followed by incubation at 37 °C for 24 hours. Colonies with yeast morphological characteristics were purified by conducting streak culture for single colonies and stored at -20 °C in Potato Dextrose Broth (PDB) supplemented with 30% v/v glycerol, before being characterized for the survival test at low pH condition and survival at high concentration of deoxicholic acid as well as being tested for transformation of cholic acid into deoxicholic acid, for assimilating cholesterol in order to see the yeasts potential as novel probiotics.

Growth of Yeast Isolates at pH 7 Following Exposure to Low pH Conditions (Acidic Conditions)

One full loop of each yeast isolate was inoculated into 5 mL of Potato Dextrose Broth (PDB) and incubated at 37 °C for 24 hours to obtain cell density of approximately 10^8 cells/mL (according to McFarland scale). A volume of 100 µL of this suspension were then transferred into 900 µL of the same medium with various pH levels, i.e., pH 2, 3, and 4, and then incubated for 3 hours at 37 °C in a water bath, centrifuged at 7,000 rpm until a pellet-form was obtained. The pellet was then washed twice with 300 µL saline solution. The washed pellet was then re-suspended in 300 µL PDB at pH 7, from which 50 µL was transferred into 6.5 mL PDB at pH 7, incubated at 37 °C for 24 hours, and finally, the optical density of the

pellet was measured at wavelength of 660 nm (OD_{660}). The survival of the yeast in this PDB medium at pH 7 after being exposed to various low pH levels in the same medium was indicated by an increase in turbidity (OD reading) of the yeast suspensions. Triplicate experiments per isolate were carried out to obtain representative data.

Survival at High Concentration of Sodium Deoxicholic (NaDC)

A volume of 50 µL suspension of yeast isolates were inoculated to PDB that each containing three different concentrations of NaDC, i.e., 0.2 mM, 0.4 mM, and 0.6 mM. PDB without NaDC served as control. All test tubes were incubated at 37 °C for 24 hours and then measured for turbidity using a spectrophotometer at the wavelength of 660 nm (OD_{660}). Triplicates per treatment were prepared to obtain representative data. An increase in OD reading of the yeast isolates in PDB containing various concentration of NaDC indicated the yeast's survival in such medium following exposure with high level of NaDC.

***In-vitro* Evaluation of Yeast Isolate's Ability to Assimilate Cholesterol**

The ability of yeast isolates to assimilate cholesterol *in-vitro* was conducted by following the method specified by Buck and Gilliand (1994). Each isolate was initially grown in potato dextrose broth (PDB) at 37 °C for 24 hours. The yeast suspensions of 100 µL was next inoculated to 9.9 mL PDB supplemented with 2% sodium thioglycollate, 0.3% oxygall, and 0.10% cholesterol so that the total volume of each system was 10 mL, and then incubated at 37 °C for 24 hours, centrifuged for 20 minutes at 3,500 *xg* to obtain cell-free supernatant. Following these steps samples were analyzed for cholesterol residue in each culture using O-pthalaldehyde method as the indicator reagent. Un-inoculated medium served as control. The difference of cholesterol residue in the control and in the inoculated media was assumed as the amount of cholesterol assimilated by the yeast isolates.

Tests for Biotransformation of Cholic Acid into Deoxicholic Acid

This test adopted the method specified in Uni (2012). A volume of 50 µL of yeast stock culture in PDB-glycerol medium was transferred into 5 mL PDB and incubated at 37 °C for 24 hours to produce cell suspension with approximate density of 10^8 cells/mL. Subsequently, a suspension of 50 µL was grown at 37 °C for 24 hours in the same medium supplemented with cholic acid (CA). After that, 1 mL of the suspension was centrifuged at 5,000 rpm. A volume of 0.1 mL of the supernatant was subsequently transferred to a new Eppendorf tube to which 500 µL of ethyl acetate and 20 µL HCl was added and then centrifuged at 5,000 rpm for 5 minutes. Subsequently, the supernatant was evaporated at room temperature for 48 hours, and finally added with 15 µL methanol. This sample was then used for thin layer chromatography (TLC) to determine the extent of transformation. The eluent for the TLC consisted of a mixture of 10 mL cyclohexane, 15 mL ethyl acetate, and 4 mL acetic acid. Before being used, this eluent was left for 30 minutes in a chamber in order to obtain saturated vapor of the mixture in the chamber.

The TLC was run on a piece of aluminium silica gel, in a chamber previously prepared. Samples and controls (cholic acid and deoxicholic acid) amounted at 1 µL were spotted on the aluminium silica gel and dried using a hair dryer. On the completion of the TLC run, the silica gel was removed from the chamber, air-dried at room temperature, sprayed with Molibddophosporic acid, heated in an oven until black spots appeared on the silica gel, and photographed for documentation.

RESULTS AND DISCUSSION

Ten yeast isolates with potential as probiotics were successfully isolated in this study (Table 1). All yeast isolates were found to ferment glucose and produce ethanol as well as gas from this metabolism. The colony morphology of our isolates was either rough or smooth, with pseudohyphal morphology. Based on their cell morphology and biochemical evaluation, all isolates were preliminary identified as yeasts belonged to genus *Saccharomyces*. These characteristics are in line with those reported by Reis *et al.* (2014). Yeasts belonging to this genus have been reported by many previous studies to have potential to be used as food supplements

for human or animal probiotic. Pais *et al.* (2020) reported that *S. boulardii* plays important roles to maintain and control the balance of human or animal intestinal normal microbiota when included as food supplements. Besides, yeast belongs to genus *Saccharomyces* was also reported to prevent *Clostridium difficile* from infecting human digestive tract (Mills *et al.* 2018). In the recent years, the beneficial properties of such yeast strain have been extensively reviewed by Tomičić *et al.* (2016). This strain was also engineered genetically by Liu *et al.* (2016) so that the strain is able to produce several products of interest, including lysozyme which is important to improve and maintain intestinal tract health. This opens the possibility for our yeast isolates to be developed as potential and novel probiotics for cattle or human in the near future, although further evaluation is needed to elucidate their probiotic potencies.

Before being developed as novel and potential probiotics, isolates must show a high level of viability following exposure to extreme conditions of the upper part of the intestinal tract, such as low pH and high concentration of NaDC). In the actual application, such probiotic candidates must flow along this part of the intestinal tract before reaching the lower part of the intestinal canal where they normally provide beneficial effects to their hosts (Fuochi *et al.* 2015). The shown ability to transform cholic acid into deoxicholic acid must be excluded for further screening (Jia *et al.* 2018). The yeast isolates in our study showed the capability to survive exposure to low pH conditions *in-vitro* (Table 1). All isolates were found to grow in such medium conditions, although their growth was slightly inhibited due to 3-hour exposure to low pH conditions. The 3-hour incubation period was chosen in this study, because the probiotic candidates will experience three hour contact in the upper part of the intestinal tract when applied in the actual situation and this was in line with that reported by Oozer *et al.* (2006). Surprisingly, all isolates were found to survive in the exposure to pH 2 for 3 hours, although low turbidity was recorded after being incubated in new medium having pH 7 for 24 hours. The results of this study indicated that all yeast isolates may have the potential to be developed as probiotics, because under certain condition (such as empty stomach), the pH of the stomach may decrease to 2 or even 1.5 (Beasley *et al.* 2015). Knowledge about the mechanism by which microbial cells survive in a low pH medium is still limited. However, some studies reported that in order to survive under an extremely low pH medium, the cells must maintain their internal pH to be higher than that of their surroundings. (Guan *et al.* 2020) by metabolizing glucose through which they derive energy so that they can activate proton pump to exclude H^+ ions out from their cytoplasm. This process requires a significant amount of energy in the form of adenosine triphosphate or ATP (Anandakrishnan & Zuckerman 2017). Failure to maintain internal pH condition to be higher than that of cell's environment will lead to cell's death.

Table 1 Growth of yeast isolates at pH 7 after being exposed to low pH conditions

Isolate codes	Optical density at 660 nm (OD_{660})*			
	Control (pH 6.5)	pH 2	pH 3	pH 4
N1	++ (0.623 ± 0.141)	++(0.515 ± 0.064)	++(0.586 ± 0.057)	++(0.509 ± 0.092)
N2	++(0.653 ± 0.142)	+(0.328 ± 0.057)	++(0.598 ± 0.201)	++(0.588 ± 0.061)
N3	++(0.559 ± 0.160)	+(0.326 ± 0.050)	++(0.644 ± 0,177)	++(0535 ± 0.047)
N4	++(0.529 ± 0.075)	+(0.339 ± 0.040)	++(0.600 ± 0.033)	++(0.585 ± 0.128)
N5	+++(1.148 ± 0.291)	+(0.478 ± 0.136)	++(0.530 ± 0.068)	++(0.549 ± 0.185)
G1	++(0.970 ± 0.183)	++(0.553 ± 0.060)	++(0.691 ± 0.106)	++(0.620 ± 0.203)
G2	+++(1.110 ± 0.022)	+(0.445 ± 0.038)	++(0.664 ± 0.340)	++(0.747 ± 0.068)
G3	++(0.865 ± 0.122)	++(0.610 ± 0.140)	++(0.750 ± 0.215)	++(0.582 ± 0.197)
G4	+++(1.033 ± 0.051)	++(0.541 ± 0.154)	++(0.756 ± 0.138)	++(0.893 ± 0.029)
G5	+++(1.061 ± 0.049)	++(0.514 ± 0.122)	++(0.654 ± 0.098)	++(0.628 ± 0.086)

Notes: * = Each value ± standard deviation in Table 1 is an average of triplicate measurements of optical densities (absorbance/A) of the cell suspensions which indirectly measure the growth of the cells in the low pH medium. The growth indication follows the following criteria: - = OD of < 0.1 (Sensitive to low pH conditions); + = OD of 0.1 - 0.5 (Slightly resistant to low pH conditions); ++ = OD of 0.5 - 1.0 (Resistant to acid conditions); +++ = OD of > 1.0 (Highly resistant to acidic conditions).

In addition to resistance to acidic conditions, the yeasts isolates were also found to survive in medium containing high concentration of NaDC (Table 2), which is also a good indication for their possible use as potential probiotic candidates. On their way to colon, the probiotic must experience an extreme condition in the small intestine where high concentration of bile is secreted by the liver (Darilmaz 2013). Bile is toxic for microbes because of its function as biodetergent in the intestinal tract of human and other animals (Urdaneta & Casadesús 2017). The effects of this bile for bacterial cells can be fatal (cell death) as it can cause disintegration of the cell membrane system (Urdaneta & Casadesús 2017). Other mechanism by which bile can disturb cell function is related to DCA uptake by the cells. Once exposed to neutral pH within the cell cytoplasm, the DCA will be in a undissociated form and it is difficult for the cells to pump it out using proton motive force mechanism (Chatterjee *et al.* 2004). Accumulation of this compound in the cell can cause significant damage on DNA and this will result in cell death (Urdaneta & Casadesús 2017).

All yeast isolates showed resistance to various levels of NaDC as indicated by high OD reading (ranging from 0.5 to 1.0) (Table 2). Although it was not investigated in the present study, tolerance property to NaDC exhibited by our yeast isolates might be related to glycosidase activity (Kwun *et al.* 2017), the composition of membrane protein and fatty acid (Gueimonde *et al.* 2005), and exopolysaccharide production (Nguyen *et al.* 2020). The ability of cells to prevent damage on their cell membrane and DNA during exposure to high level of NaDC also plays important roles to survive in such condition (Merritt & Donaldson 2009). According to Kusada *et al.* (2021) enzyme activity (bile salt hydrolase activity) that converts the physicochemical properties of the bile salt reduces toxicity of this compound to the cells.

The potential of the promising isolates to assimilate cholesterol *in-vitro* is shown in Figure 1. All isolates showed their ability to assimilate cholesterol in the medium with various degrees of degradation rates (between 18 - 76% reduction/24 hours incubation). When compared to control treatment (where the reading was constant or no reduction in cholesterol in the medium), these values were statistically significant ($P < 0.05$). Only two isolates (N1 and N5) assimilated cholesterol at the rate of less than 50% (Fig. 1). The others assimilated cholesterol at the rate of between 52% and 76%, relative to control, indicating that these isolates showed potential for further probiotic development.

The data presented in Figure 1 is in line with that mentioned by previous studies which found that some yeast species modified fat and its derivatives in the process of fermentation. Aloglu *et al.* (2015) reported that yeast, including *Saccharomyces cerevisiae,* had the ability to metabolize sterol. Other yeast species, such as *Candida lipolytica* was also reported by Ali *et al.* (2010) to metabolize fats. In addition to yeasts, bacterial probiotics (lactic acid bacteria) have also been reported by Tokatli *et al.* (2015) as having the capability to assimilate cholesterol. This yeast characteristic is important to reduce cholesterol absorption in the small intestine, which leads to reduced level of blood cholesterol.

Table 2 Growth of yeast isolates in a medium containing various levels of NaDC

Isolate codes	Optical density at 660 nm (OD_{660})*			
	Control	0.2 mM NaDC	0.4 mM NaDC	0.6 mM NaDC
N1	++(0.89 ± 0.24)	++(0.97 ± 0.25)	+++(1.20 ± 0.26)	+(0.58 ± 0.03)
N2	++(0.81 ± 0.21)	++(0.84 ± 0.34)	++(0.67 ± 0.10)	+(0.34 ± 0.16)
N3	++(0.87 ± 0.10)	++(0.74 ± 0.05)	++(0.85 ± 0.05)	+(0.29 ± 0.19)
N4	++(0.88 ± 0.90)	++(0.86 ± 0.01)	++(0.82 ± 0.06)	++(0.69 ± 0.29)
N5	++(0.99 ± 0.09)	++(0.84 ± 0. 04)	+++(1.10 ± 0.14)	+(0.21 ± 0.02)
G1	++(0.55 ± 0.09)	++(0.75 ± 0.11)	++(0.79 ± 0.06)	+(0.49 ± 0.17)
G2	++(0.59 ± 0.06)	++(0.81 ± 0.01)	+++(1.03 ± 0.09)	++(0.54 ± 0.37)
G3	++(0.73 ± 0.19)	++(0.98 ± 0.21)	+++(1.09 ± 0.23)	+(0.29 ± 0.21)
G4	++(0.66 ± 0.10)	++(0.87 ± 0.35)	++(0.95 ± 0.08)	+(0.44 ± 0.08)
G5	+++(1.04 ± 0.06)	++(0.92 ± 0.04)	+++(1.02 ± 0.14)	+(0.43 ± 0.38)

Notes: * = Each value ± standard deviation in Table 2 is an average of triplicate measurements of optical densities (absorbance/A) of the cell suspensions which indirectly measure the growth of the cells in various levels of NaDC. The growth indication follows the following criteria: - = OD of < 0.1 (Sensitive to NaDC); + = OD of 0.1 - 0.5 (Slightly resistant to NaDC); ++ = OD of 0.5 - 1.0 (Resistant to NaDC); +++ = OD of > 1.0 (Highly resistant to NaDC).

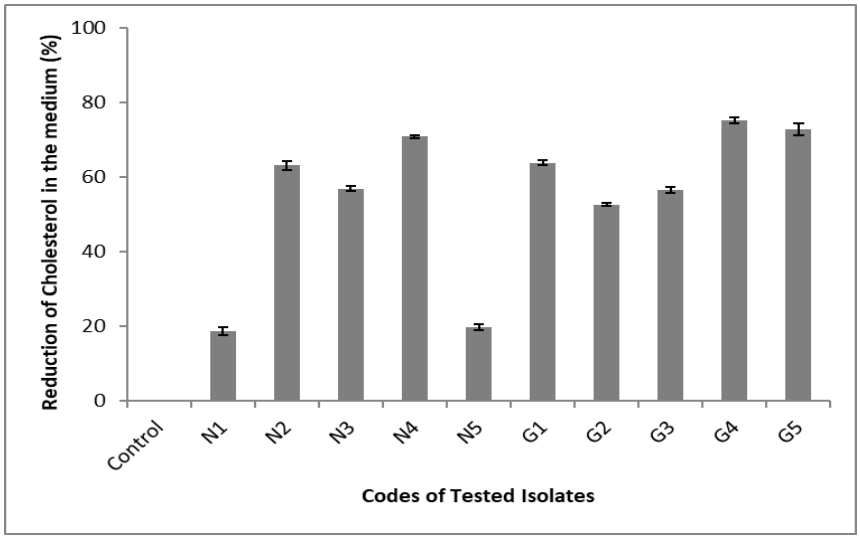
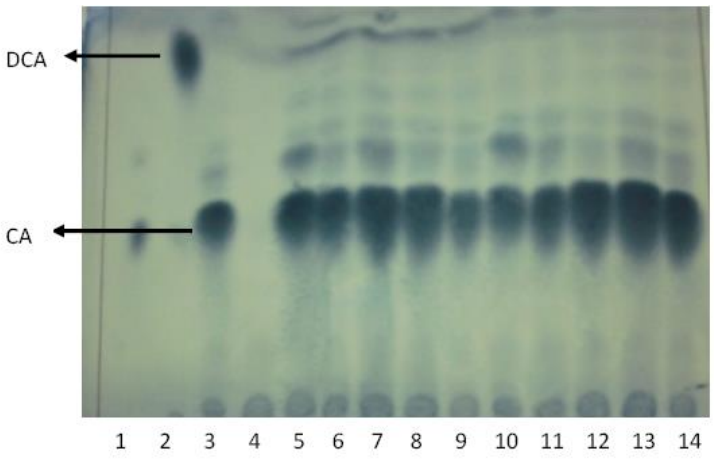


Figure 1 Percentage of cholesterol reduction in the medium after being inoculated with isolates of yeast and incubated for 24 h in PDB medium supplemented with cholesterol.
Note: Values in the figure ± standard deviation bars are average value of triplicates.

Microbial isolates intended to be developed as potential probiotics should not transform cholic acid into deoxicholic acid in the intestinal track of their hosts. According to Ajouz *et al.* (2014) and Farhana *et al.* (2016), the accumulation of deoxicholic acid in the digestive track has been suspected to induce colon cancer. Although yeast has the potential and has positive properties to be a probiotic candidate, the yeast will be rejected if it has the ability to transform cholic acid into deoxicholic acid. In our present study, the 10 yeast isolates (Table 1 & 2) were evaluated for biotransformation of cholic acid into deoxicholic acid. The chromatogram of this evaluation indicated that none of the yeast isolates in our study transformed cholic acid into deoxicholic acid (Fig. 2).



Lane 1 and 3 are deposited with colic acid (CA)
Lane 2 is deposited with Sodium Deoxicholic acid (NaDC)
Lane 4 is deposited with methanol
Lane 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 are respectively deposited with cell-free supernatants of isolates N1, N2, N3, N4, N5, G1, G2, G3, G4, and G5.

Figure 2 A chromatogram of test for biotransformation of cholic acid into deoxicholic acid for the 10 yeast isolates in our study.

The chromatogram confirmed that all yeast isolates obtained in our present study are safe and have the possibility to be developed as potential probiotics without harming human or cattle as their hosts. In the intestinal track, biotransformation of cholic acid into deoxicholic acid is commonly performed by *Clostridium scindens* (Guzior & Quinn 2021). High concentration of deoxycholic acid in the intestinal tract was reported by Wu *et al.* (2018) as having significant effect on the enlargement of colorectal tumor.

CONCLUSION

All 10 yeasts isolates used this study were shown to be resistant to low pH conditions and high concentration of bile salt (NaDC). The isolates were also found to reduce cholesterol content *in-vitro* at the rate of between 18 - 76% following 24 hours incubation. Besides these positive attributes, none of the isolates transformed cholic acid into deoxicholic acid, indicating that they all have probiotic potential and are safe to be developed as novel probiotics in Bali, either for human or cattle.

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