CHAPTETR 7

HYPOCHOLESTEROLEMIC ACTIVITY OF INDIGENOUS PROBIOTIC ISOLATE *SACCHAROMYCES CEREVISIAE* ARDMC1

Chapter 7

Hypocholesterolemic activity of indigenous probiotic isolate *Saccharomyces cerevisiae* ARDMC1

7.1. Abstract

The aim of this study was to investigate probiotic attributes of *Saccharomyces cerevisiae* ARDMC1 isolated from traditional rice beer starter cake and its hypocholesterolemic effects on Wistar rats fed with high cholesterol diet. The indigenous isolate ARDMC1 showed potential probiotic characteristics such as tolerance to simulated gastrointestinal stress conditions, autoaggregation properties and adhesion to intestinal epithelium Caco-2 cell line. In addition, ARDMC1 isolate exhibited *in vitro* cholesterol assimilation properties in media supplemented with cholesterol. Furthermore, administration of probiotic isolate to rats fed with hypercholesterolemic diet resulted in significant reduction of serum total cholesterol, low-density lipoprotein cholesterol and triglyceride at the end of 42 days. The present study envisages ARDMC1 as a promising starter culture for the preparation of functional foods with properties to combat cardiovascular diseases.

7.2. Introduction

Cardiovascular disease (CVD) and its related complications are triggered by elevated serum cholesterol levels and are considered as the leading causes of death worldwide. According to a report published by the World Health Organization (WHO), 17.5 million people died from CVDs in 2012, representing 31% of all global deaths and morbidity is expected to increase to 23.3 million by 2030 [1, 2].

Several forms of therapy have been reported to prevent CVDs; however, the resources available for its management in low and middle- income countries are limited. Statins are the most well known hypolipidemic drugs which act as inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase essential for the metabolic pathway producing cholesterol and other isoprenoids in the body [3]. However, side effects associated with statins like myalgias and muscle weakness, increased fatigue, reduced energy, deteriorating hyperglycemia and risk of new onset diabetes possess a greater threat to human health [4, 5].

Many studies showed that probiotics or products containing them impart various health benefits that include prevention of cardiovascular diseases (CVDs) and enhancing general wellness of consumers [6, 7]. Probiotics are 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' [8]. Recent studies also reported that the microbial communities for probiotics have revealed some interesting attributes of yeasts and functional foods prepared with probiotic yeasts reduce the levels of lipids in the serum of rats fed with high cholesterol diet [9]. Yeast cells from food and animal origin were also reported to remove cholesterol from media, under simulated conditions that mimics the gastrointestinal tracts of monogastric animals [10, 11].

To address the alarming burden of CVDs, a cost-effective and affordable alternative strategy is required to reduce the CVDs related risk factors. A recent approach for lowering cholesterol and hence minimizing the risk of CVDs is the use of probiotic and prebiotic based functional or health foods which modulate the gut microbial ecosystem or their metabolic products [12, 13]. In recent studies, antiatherosclerotic effects of traditional fermented foods from Asia have gained lots of attention [14]. Keeping this in view, *Saccharomyces cerevisiae* ARDMC1 was investigated for *in vitro* probiotic attributes and cholesterol lowering properties in rat fed with high cholesterol diet. In the present study, *Saccharomyces cerevisiae* ARDMC1 was isolated from starter culture cake of *Apong*, a traditional rice beer of the *Mishing* tribe of Assam, India. *Apong* has a socio- cultural status as a popular alcoholic beverage inimitable to the *Mishing* community and reported to have various health promoting benefits [15].

In this study, *Saccharomyces cerevisiae* ARDMC1 was isolated from starter culture cake of *Apong*, a traditional rice beer of the *Mishing* tribe of Assam, India. *Apong* has a socio- cultural status as a popular alcoholic beverage inimitable to the *Mishing* community and gifted with various health promoting properties [9]. The present study deals with the evaluation of *in- vitro* probiotic properties and hypolipidemic effects of ARDMC1 in an in *vivo* rat model system.

7.3. Materials and methods:

7.3.1. Yeast and bacterial strains, culture media, and growth conditions

In this study, *Saccharomyces cerevisiae* ARDMC1 (out of 23 isolates) was isolated from rice beer starter culture of Assam, India and selected based upon promising probiotic attributes and their *in-vitro* cholesterol removal properties. The probiotic reference strain *Saccharomyces boulardii* was isolated from marketed probiotic drug (Lupin Laboratories Ltd, India). *Salmonella enterica typhemurium* MTCC 1252 was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The yeast strains, *S. cerevisiae* ARDMC1 and *S. boulardii* were grown in YMB (Yeast and Mould Broth, HIMEDIA, India) medium under shaking conditions at 30ºC for 48 h, whereas *Salmonella enterica typhemurium* was grown in nutrient agar medium (HIMEDIA, India) at 37 ºC for 24 h.

7.3.2. Molecular Identification of isolates

The 5.8S-ITS rDNAs of yeast isolates ARDMC1 and *S. boulardii* were amplified using the primers ITS-1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS-4 (5´-TCCTCCGCTTATTGATATGC-3´) [16]. The D1/D2 domain of the 26S rDNA gene was amplified using the primer pair NL1 (5´-GCATATCAATAAGCGGAGGAAAAG-3´) and NL4 (5´-GGTCCGTGTTTCAAGACGG-3´) [17]. The PCR amplification was performed in an Eppendorf Thermocycler according to a protocol developed in our laboratory with slight modifications [18]. Briefly, amplification parameters consisted of an initial denaturation step of 3 minutes at 95 ºC, followed by 30 cycles of 95 ºC for 30 seconds, primer annealing for 30 seconds at 58 ºC, elongation for 1 minute at 72 ºC, and final extension of 10 minutes at 72 ºC for 1 cycle. The amplified PCR product was purified and subjected to automated DNA sequencing using 3130 Genetic Analyzer (Applied Biosystem, Switzerland). The phylogenetic tree was generated by Neighbor-Joining (NJ) method using MEGA 5.05. The sequences obtained were submitted to NCBI (National Center for Biotechnology information) Genbank (http:// www.ncbi.nlm.nih.gov/genbank).

7.3.3. *In vitro* gastrointestinal stress tolerance test

Tolerance to simulated gastrointestinal conditions was evaluated according to a method developed by Maragkoudakis et al. [19] with some modifications. Briefly, cells from a 48 h culture were harvested by centrifugation at 6000 x g for 5 minutes at 4 ºC, washed once with phosphate buffered saline (PBS) solution with pH 7.4, before being re-suspended into simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) at a cell density of 10^8 colony forming units (CFU)/mL. SGF was prepared by supplementing sterilized PBS, pH 2, 3 and 4 with pepsin to a final concentration of 3 g/l. SIF was prepared by supplementing sterilized PBS, pH 6.8 and 8 with pancreatin (Sigma Aldrich, USA) to a final concentration of 1 g/l and 0.3 g/l bile salt mixtures. The resistance of isolates was evaluated by counting viable colony on YMB agar plates after 0, 1, 2 and 3 h for SGF and 0, 1, 2, 3, 4 h for SIF experiments, respectively.

7.3.4. Hydrophobicity

The hydrophobicity of isolate was assessed by following the method of Rosenberg, 2006 [20]. Cells from a previously grown culture were harvested and washed twice with PBS, pH 7.4. Cell count was adjusted approximately to 10^9 CFU/mL. 2 mL of cell suspension was mixed with equal volume of n-hexadecane by vortexing for 2 minutes. The aqueous and the organic phases were allowed to separate by keeping the mixture undisturbed for 1 h. After that, the aqueous layer was gently pipetted out and OD_{600} was measured. The cell surface hydrophobicity was calculated as:

$$\text{Hydrophobicity}(\%) = \left(\frac{Abs_{initial} - Abs_{final}}{Abs_{initial}}\right) \times 100 \quad (1)$$

Where $Abs_{initial}$ represents initial absorption before mixing and Abs_{final} represents final absorption after mixing with n-hexadecane.

7.3.5. Autoaggregation and coaggregation

Autoaggregation capacity of the isolate was evaluated using previously described methods [21]. Briefly, 4 mL of cell suspension (10^9 CFU/mL) was vortexed for 10 second and incubated at 37ºC. After 4 h, a 100 µL aliquot was taken out from the upper surface, mixed with 900 µL of PBS (pH 7.4) and OD_{600} was measured. Autoaggregation percentage was calculated as:

$$\text{Autoaggregation (\%)} = \left(1 - {}^{A_t}/_{A_0}\right) \times 100 \quad (2)$$

Where A_t= absorbance at 4 h and A_0= absorbance at 0 h.

For the coaggregation assay, a mixed culture was prepared by mixing equal volume (2 mL) of the isolate and pathogenic indicator (*Salmonella enterica typhemurium*, MTCC 1252) cell suspension (~10^9 CFU/mL) by vortexing for 10 seconds. Control tubes were set up at the same time, containing 4 mL of each bacterial suspension. The absorbance (A_{600}) of the suspensions was measured followed by mixing and 4 h of incubation at room temperature. Samples were taken in the same way as in the autoaggregation assay. The percentage of co-aggregation was calculated using the equation of Handley et al. [22].

$$\text{Coaggregation (\%)} = \frac{\{(Ax+Ay/2)\}-A(x+y)}{Ax+Ay/2} \times 100 \quad (3)$$

Where x and y represent each of the two strains in the control tubes, and (x + y) represents the mixture.

7.3.6. Probiotic adhesion to Caco-2 cells

The human colorectal adenocarcinoma Caco-2 cell line was procured from the National Centre for Cell Science (NCCS), Pune, India. The cell line was routinely grown and maintained in minimal essential medium (MEM) supplemented with 20% heat- inactivated fetal bovine serum (FBS) [23]. Media and reagents were purchased from Sigma (India) and Gibco® (Life Technologies).

The adhesion study was performed using previously described method of García-Cayuela et al. [24]. Briefly, Caco-2 cells were seeded at a concentration of 1×10^4 cells/mL in 24-well tissue culture plates (NEST Biotechnology) and grown for 14 days to achieve about 80 % confluence at 37 °C in a humidified atmosphere containing 5% CO_2. The yeast cells grown previously were harvested by centrifugation (5000 rpm for 5 min), washed twice with PBS 1X and resuspended in MEM without antibiotic supplementation at a concentration of about 10^8 CFU/mL. For adhesion assay, Caco-2 cell monolayers were washed to remove media containing antibiotic and inoculated with fresh yeast cell suspensions (yeast cells: Caco-2 cells at a ratio of 10:1). After an incubation period of 1 h, media was discarded and wells were gently washed three times with PBS buffer to remove non-adhering probiotic cells. Finally, Caco-2 monolayers were trypsinized with 0.25% trypsin-EDTA

solution (Sigma) and the number of adherent isolates was determined by serial dilution plating on YMA. All the experiments were performed in triplicate. Adhesion data were expressed as the percentage of yeast cells adhered compared to the total inoculum added (CFU yeast cells adhered/CFU yeast cells added). For visualization of adhesion, Caco-2 cell monolayers were washed three times with PBS, dried in air and adherent yeast cells were observed in microscope (EVOS FL cell imaging system, ThermoFisher) under 20X after fixing with 3 % paraformaldehyde.

7.3.7. Assimilation of cholesterol by isolates

The ability to assimilate cholesterol was performed following the method of Lye, Rahmat-Ali and Liong, 2010 [25] with some modifications. Yeast isolates were inoculated to YM broth supplemented with water-soluble cholesterol (Polyoxyethanyl-cholesterylsebacate, Sigma) at a concentration of 50 µg/mL and 0·3% Ox-bile followed by incubation at 37 °C. The presence of cholesterol in the spent broth was determined using a colorimetric method [26]. The attachment of cholesterol particle onto cell surface was visualized under scanning electron microscopy (SEM). The cell pellet obtained after centrifugation in the previous step was fixed with 2.5% glutaraldehyde for 6 h. The samples were then centrifuged and the pellet was resuspended in 1X PBS, pH 7.4 for 1 h containing 1% osmium tetroxide (Sigma–Aldrich). Further cells were dehydrated in graded concentrations of ethanol. Then specimens were platinum coated using JEOL JFC-1600 auto fine coater and observed under SEM (JEOL model JSM-6390 LV) at 20 kV.

7.3.8. Cholesterol-lowering ability of isolates in Wistar rat model

Cholesterol lowering ability was tested following a method reported by Kumar, Grover and Batish, 2011 [27] with slight modifications. Briefly, a total of thirty adult male Wistar rats (mean body weight 150 gram) used in this study were maintained at Defense Research Laboratory, Tezpur, India (Animal ethics approval no: 1AEC/01/2015) and housed six animals per cage under a constant 12 h light–12 h dark cycle with a controlled temperature at 25 °C and a relative humidity of 56–60%. Both *S. cerevisiae* ARDMC1 and *S. boulardii* were separately mixed with high fat diets (HFD) to achieve a final concentration of approximately 10^8 CFU/g. The CFU/g of diet was counted by suspending 1 gram of diet in 9 mL of phosphate buffer saline,

and then the appropriate dilutions were made and plated on YMB agar to determine the exact CFU/g of diet. All animals were acclimatized by feeding on a basal diet for one week prior to experiment. The animals were divided into five experimental groups (i) normal diet (ND), (ii) HFD, (iii) HFD with statin (Atrovastatin Tablets IP, Macleod, India), (iv) HFD with *S. boulardii* and (v) HFD with *S.cerevisiae* ARDMC1. All rats had free access to water and their specific diets (20g/100g body weight per day). The body weights of the animals were measured weekly. The experimental high fat diet was formulated and fed to the animals for 42 days with slight modification of the method reported earlier [27]. The HFD contained cholesterol (0.5%), dalda (30%), refined soy oil (10%), and wheat flour (50%) as major constituents.

7.3.9. Analysis of serum lipid profile

For the collection of blood samples animals were fasted overnight and in the next day blood was collected from the retro- orbital sinus and preserved in pre-chilled tubes at -20 ºC. For serum lipid analysis blood samples were collected at 21 and 42 days of feeding trial. The collected blood samples were centrifuged at 2000 x g for 15 minutes at 4 °C. The serum obtained was analyzed for total cholesterol (TC), triglycerides (TAG), and HDL-cholesterol using commercial enzymatic kits (Autopak, M/s Siemens Diagnostics Limited). Friedewald's equation [28**]** was applied to analyze the following other plasma lipid fractions:

(1) LDL-cholesterol = total cholesterol - HDL-cholesterol - (TAG/5). All the concentrations are given in mg/dL.

(2) VLDL-cholesterol = the quotient (TAG/5) is used as an estimate of VLDL-cholesterol.

Atherogenic index (AI) was calculated according to the formula AI= (Total cholesterol-HDL-C)/HDL-C and LDL-C/HDL-C ratio was calculated as the ratio between plasma LDL-C and HDL-C as proposed by Harnafi et al. [29].

7.4. Results and discussions

7.4.1. Molecular Identification of isolates

The use of yeast as potential probiotics has gained enormous interest in food pharmaceutical industries. Yeast contributes a significant role in the production of some cheeses and fermented milk [30 and 31]. Fig. 7.1A depicts the amplicons obtained from PCR reactions. The sizes of the amplicons were around 850 bp (5.8S-ITS) and 560 bp (D1/D2 domain) for ARDMC1, which corroborate with the expected amplicon size of *Saccharomyces* strain reported previously [32, 33]. The sequences obtained from 5.8S-ITS rDNA and domain D1/D2 were used to construct the phylogenetic tree (Fig. 7.1B and 7.1C) using Neighbor-Joining method and showed maximum similarity to *S. cerevisiae* Sc19 and *S. cerevisiae* QWF respectively. The gene sequences of 5.8S-ITS rDNA (KF414969) and the D1/D2 domain of the large subunit of 26S rDNA (KP233782) were submitted to NCBI GenBank. The probiotic reference strain *S. boulardii* used in this study was confirmed based on sequencing of 5.8S-ITS rDNA and 26S rDNA.

7.4.2. Simulated gastrointestinal tract and bile tolerance

The low pH of the stomach and intestinal fluids (e.g. bile and pancreatic juice) towards the distal part of the gastrointestinal tract is inhibitory to most of the microorganisms. The probiotic candidate must withstand these harsh conditions to exhibit health benefits on the host. In the present study, ARDMC1 yeast isolate exhibited considerable tolerance to gastrointestinal stress conditions, proving their suitability as putative probiotics. Previous studies also reported that probiotic yeasts can survive under gastrointestinal stress conditions [34]. Table 7.1 represents the survivability of ARDMC1 and probiotic reference strain *S. boulardii* after exposure to SGF and SIF conditions. When exposed to pepsin supplemented SGF of pH 2 for 3 h, the yeast cell (ARDMC1) count was reduced to ~1.5 log units or 20.79%, whereas *S. boulardii* showed a decrease of 2.16 log units or 26.6% after exposure to the same conditions. The viability of ARDMC1 at pH 3 and 4 showed more than 6 log units after exposure for 3 h as compared with *S. boulardii*. However, survivability of ARDMC1 was not inhibited significantly (P <0.05) and showed considerable resistant

under simulated intestinal fluid (SIF) conditions at pH 8.0, although its viability decreased from log 8.2 to about log 6.4 CFU/mL.

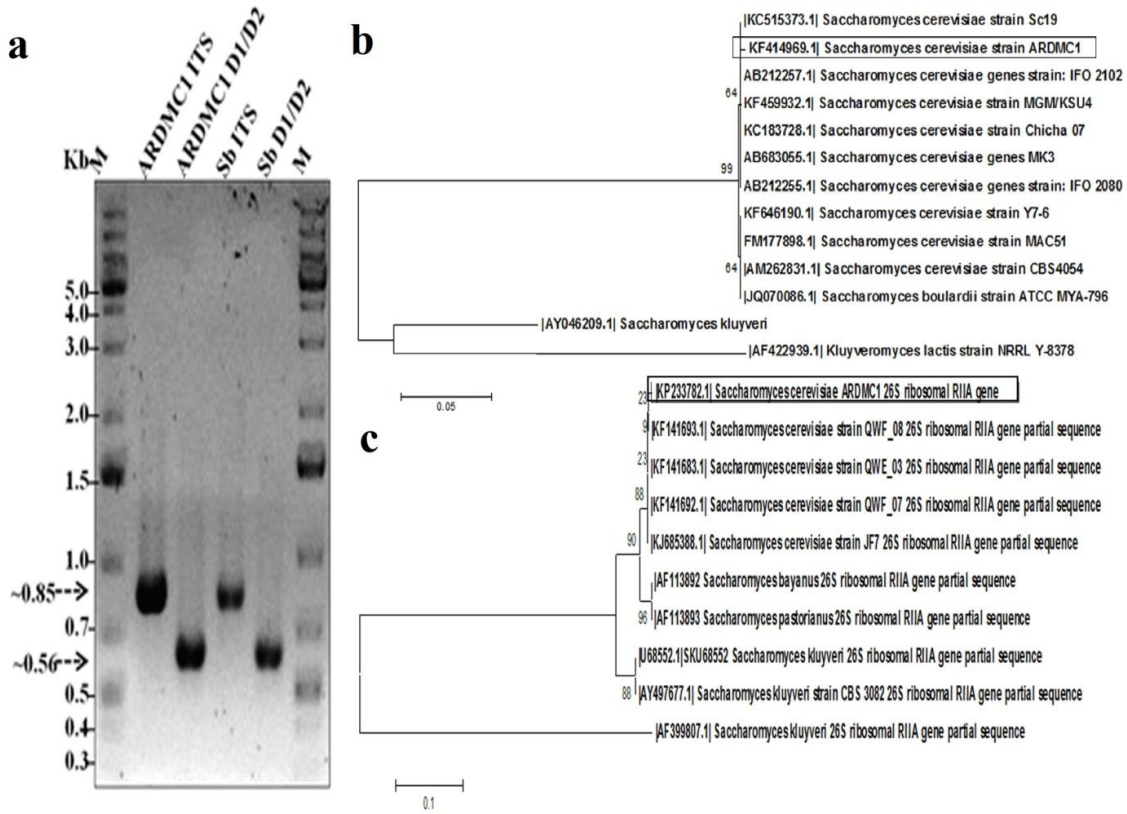

Fig. 7.1. (A) Molecular typing of Sc and Sb isolates by using ITS (internal transcribed spacer)-primer (Lane 2 and 4) and 26S rRNA gene D1/D2 region (Lane 3and 5); Lane 1 & 6 indicates GeneRulerTM 1 Kb Plus DNA ladder (Fermentas); Phylogenetic tree showing *S. cerevisiae* ARDMC1 with closely related species based upon (B) 5.8S ITS rRNA and (c) D1/D2 26S rRNA sequences. Bootstrap values (1,000 replicates) are indicated at branch nodes.

Table 7.1(A) Simulated gastric fluid tolerance test

Time (h)	pH 2.0 (SGF)		pH 3.0 (SGF)		pH 4.0 (SGF)	
	ARDMC1	Sb	ARDMC1	Sb	ARDMC1	Sb
0	7.63 ± 0.405^{a}	8.10 ± 0.54^{a}	7.86 ± 0.169^{a}	7.79 ± 0.34^{a}	7.78 ± 0.34^{a}	7.91 ± 0.172^{a}
1	6.13 ± 0.721^{b}	6.62 ± 0.359^{b}	6.06 ± 0.015^{bd}	6.176 ± 0.28^{b}	6.69 ± 0.42^{b}	6.24 ± 0.51^{b}
2	6.321 ± 0.45^{b}	6.28 ± 0.55^{c}	6.56 ± 0.412^{c}	6.193 ± 0.151^{b}	7.052 ± 0.6^{b}	6.68 ± 0.43^{b}
3	6.043 ± 0.06^{b}	5.94 ± 0.084^{c}	6.18 ± 0.413^{cd}	6.26 ± 0.345^{b}	6.27 ± 0.042^{b}	5.84 ± 0.34^{b}

Table 7.1(B) Simulated intestinal fluid tolerance test

Time (h)	pH 6.8		pH 8.0	
	ARDMC1	Sb	ARDMC1	Sb
0	7.92±1.43[a]	7.48±0.44[a]	8.24±0.75[a]	8.07±0.94[a]
1	6.62±0.40[a]	6.20±0.20[a]	6.27±0.15[a]	5.96±0.26[bc]
2	6.90±0.48[a]	6.00±0.19[a]	6.77±0.64[a]	6.31±0.34[ac]
3	6.49±0.25[a]	6.10±0.21[a]	6.12±0.36[a]	6.20±0.19[ac]
4	6.58±0.26[a]	6.59±0.17[a]	6.47±6.47[a]	6.57±0.47[ac]

Viability of isolates under SGF (Simulated gastric fluid) at pH 2.0, pH 3.0 and pH 4.0 (Table 7.1A) and SIF (Simulated intestinal fluid) at pH 6.8 and pH 8.0 (Table 7.1B). Values are represented as mean ± S.D, n =3. Different letters along the same column represent significant difference (P <0.05).
Abbreviation: ARDMC1 : *S. cerevisiae* ARDMC1*;* Sb: *S. boulardii*

7.4.3. Hydrophobicity, autoaggregation and coaggregation

The hydrophobic properties are correlated to the adhesion of probiotic bacteria, whereas autoaggregation and coaggregation properties enable probiotics to form a barrier against colonization of pathogens in the intestinal mucosa [35]. Hydrophobicity indices of ARDMC1 and *S. boulardii* were found to be 61.4 ± 0.10% and 58.59 ± 0.2.25% respectively. The auto-aggregation capability of ARDMC1 and *S. boulardii* did not differ significantly (P <0.05) and found to be 43.19 ± 0.90% and 40.40 ± 0.93%, respectively. Both ARDMC1 and *S. boulardii* could coaggreagate *Salmonella enterica typhemurium* up to 54.47 ± 0.02 (Table 7.2).

7.4.4. Microbial adhesion to Caco-2 cells

The adhesion and colonization to the intestinal mucosa is an essential criterion for probiotic microorganisms to enhance the immune system and impart health benefits on the host. The adhesion assay was performed using well established Caco-2 intestinal cell line from human colonic adenocarcinoma. The differences in the adhesion abilities of yeast cells might be strain specific as reported earlier [36]. Microscopic observation showed adhesion between Caco-2 cell culture and isolated strain as depicted in Fig. 7.2A, 2B and 2C. The ARDMC1 strain was also examined quantitatively for its capability to adhere to Caco-2 cells using *S. boulardii* as a reference strain. The adhesion ability of probiotic reference strain *S. boulardii*

(38.34%) was found to be higher as compared with ARDMC1 (30.43%) as shown in Fig. 7.2D. Our results suggest that both the yeast strains have significant adhesive properties, but adhesion of *S. boulardii* to Caco-2 cell line was found to be more in comparison to *S. cerevisiae* ARDMC1, in spite of showing less autoaggregation and hydrophobic properties than the latter. These findings imply that adhesion properties of probiotic yeasts are not correlated to hydrophobicity. This finding is in accordance with the work of Martins et al. 2004 [37].

Table 7.2. Autoaggrgation, coaggregation and hydrophobicity of the isolate ARDMC1 and Sb

Isolates	Autoaggregation (%)	Co-Aggregation (%)	Hydrophobicity(%)
ARDMC1	43.19 ± 0.90^{b}	44.41 ± 0.005^{a}	61.4 ± 0.1^{a}
Sb	40.40 ± 0.93^{b}	54.47 ± 0.024^{a}	$58.59 \pm 0.\ 2.25^{a}$

Values are represented as mean ± S.D, n =3.

ARDMC1 : *S. cerevisiae* ARDMC1*;* Sb: *S. boulardii*

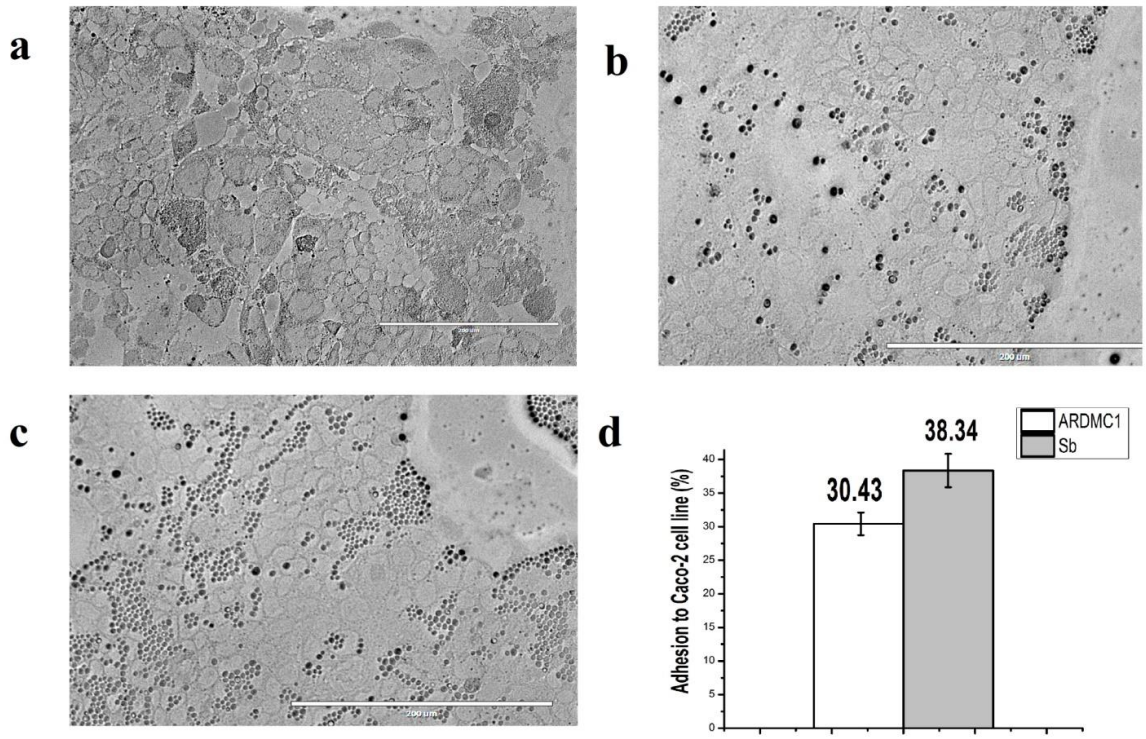

Fig. 7.2. Adhesion to Caco-2 cell line as observed under inverted microscope (20X), a) Control, b*) S. cerevisiae* ARDMC1, c) *S. boulardii* and d) % Adhesion of probiotic isolate to Caco-2 Cell line

7.4.5. In vitro assimilation of cholesterol and scanning electron microscopic studies of cholesterol binding to cellular surface

The cholesterol assimilation efficiency of spent broth and cells of ARDMC1 and *S. boulardii* from culture media is presented in Fig. 7.3. The concentration of cholesterol determined by spent broth decreased up to 20.76 ± 2 µg/mL that have been associated with the significantly (P <0.05) increased amount of cholesterol (33.69 ± 1.00 µg/mL) detected in the resuspended cells of ARDMC1 after 72 h of incubation (Fig. 7.3C). Contrary to this, *S. boulardii* could assimilate a little amount of cholesterol (8.62 µg/mL) after 72 h of incubation (Fig. 7.3F). The cholesterol assimilation of ARDMC1 yeast cells was found to be higher as compared with *S. boulardii*, isolated from the marketed probiotic drug which is in accordance with the findings of Psomas et al. [11]. Scanning micrographs also showed that cholesterol particles adhered to the cellular surface of yeast cells (Fig. 7.3A, B, D and E). The bounded cholesterol particles on the cell surfaces resulted in the roughness of cell wall. The capability of cholesterol-binding appeared to be strain specific [32].

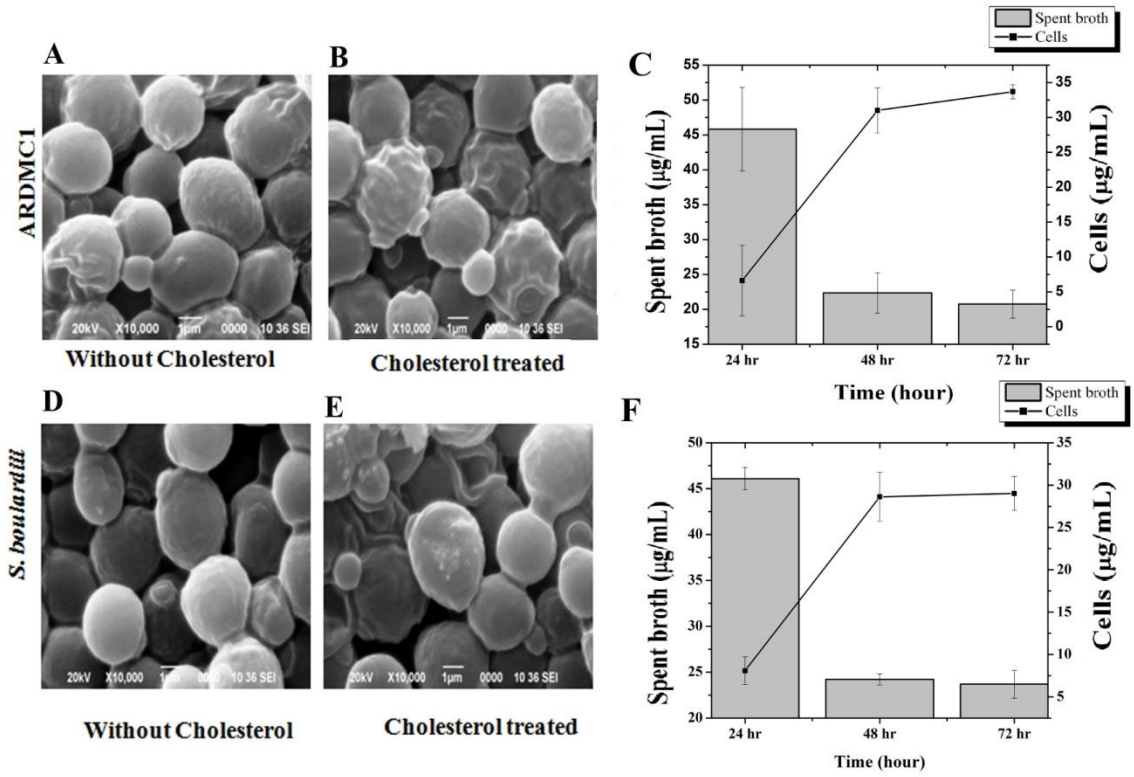

Fig. 7.3. (A) SEM image of *S. cerevisiae* ARDMC1grown in YM broth without cholesterol, (B) *S. cerevisiae* ARDMC1 grown with cholesterol, (C) cholesterol assimilation by *S. cerevisiae* ARDMC1 spent broth and resting cells, (D) SEM image

of *S. boulardii* grown in YM broth without cholesterol, (E) *S. boulardii* grown with cholesterol, (F) cholesterol assimilation by *S. boulardii* spent broth and resting cells.

7.4.6. Cholesterol-lowering ability of probiotic strains in Wistar rat model

S. cerevisiae ARDMC1 and *S. boulardii* were evaluated for their cholesterol-lowering capability under *in vivo* conditions using Wistar rat model system. As shown in table 7.3, the levels of triglyceride, LDL-C, VLDL-C and total cholesterol of HFD group were found to be significantly higher (P <0.05) than those of HFD+statin, HFD+ARDMC1 and HFD+Sb groups after 42 days of feeding trial. In contrast to the control group (HFD), the probiotic supplemented group (HFD + ARDMC1) showed lower levels of TC (62.75 ± 1.34 mg/dL), TG (122.70 ± 10.04 mg/dL), and LDL-C (21.71 ± 0.18 mg/dL) at the end of 42 days. As anticipated, HFD + Statin treatment group significantly lowered levels of serum TC, TG, and LDL-C levels to 65.83, 112.90, and 27.95 mg/dL, These values were not significantly different from those of HFD+ARDMC1 and HFD+Sb groups. Moreover, in probiotic- supplemented groups the atherogenic index (AI) and LDL-C/HDL-C ratios were significantly lower than the HFD group. There is a dearth of published information on hypocholesterolemic effects of yeast cells and few attempts have been made to assess the possible cholesterol- lowering mechanisms based on *in vivo* experiments [27 and 33]. At the end of the study, the mean body weight of the normal diet group was found to be significantly lower than the other treatment groups. The mean body weights of probiotic- treated groups were similar to the statin- treated group.

Low HDL-C and elevated levels of LDL-C, VLDL-C, TC and TGA are associated with the inception of CVD [38] and it is important to keep them in the threshold level. Cholesterol lowering by yeasts in *in vitro* conditions is due to the uptake of cholesterol as by growing yeast cells [11]. Yeasts also contain β- glucans which are reported to bind to bile acids in the intestine resulting in a decrease in bile acid pool and enhance in cholesterol breakdown. Moreover, yeasts also enhance the production of short- chain fatty acids (SCFAs) which in turn reduce the synthesis of hepatic cholesterol [39]. In our study, the strain ARDMC1 that showed promising *in vitro* cholesterol assimilation activity also showed significant lowering (P <0.05) of "bad cholesterols" LDL-C, VLDL-C, TC and TGA compared to the HFD group without challenging the concentration of so-called "good cholesterol" HDL-C in

serum. Rats fed with yeast cells or yeast- supplemented functional food showed similar results in previous studies. Both atherogenic index (AI) and LDL-C/HDL-C ratio which are two important risk factors for atherosclerosis and other types of cardiovascular diseases (CVDs) [40] were found to be decreasing in the probiotic-treated groups. The body weight increase of the rats with probiotic feed- supplements may be attributed to the growth promoting effects of yeast [9].

7.6. Conclusion

Our isolate *Saccharomyces serevisiae* ARDMC1 showed potential *in vitro* probiotic and hypocholesterolemic activity. Feeding with ARDMC1 and *S. boulardii* to high fat diet fed rats significantly decreased the serum cholesterol levels. Hence it can be concluded that incorporation of ARDMC1 in functional food may induce hypocholesterolemic effects in subjects fed with those products.

7.7. Ethical approval

All rat model experiments were performed according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India with institutional approval no: 1AEC/01/2015.

Table 7.3. Effect of probiotic isolates on serum lipids level (mg/dL) and body weight (g)

	ND	HFD	HFD+statin	HFD+ARDMC1	HFD+Sb	*p* value
21 days						
HDL-C	12.16 ± 2.14	16.50 ± 2.03	13.00 ± 3.52	12.80 ± 2.17	14.25 ± 2.87	0.6644
TGA	74.27 ± 6.41^{a}	108.12 ± 11.44^{b}	87.87 ± 6.94^{c}	80.36 ± 6.35^{a}	85.63 ± 4.19^{a}	< 0.0001
TC	59.60 ± 5.37^{a}	98.88 ± 9.55^{b}	75.06 ± 7.73^{c}	81.42 ± 7.78^{c}	74.70 ± 3.76^{c}	< 0.0001
LDL-C	32.58 ± 5.84^{a}	62.86 ± 11.27^{b}	44.49 ± 9.15^{ac}	52.55 ± 7.84^{ac}	43.33 ± 7.19^{bc}	0.0001
VLDL-C	14.85 ± 1.28^{a}	21.62 ± 2.29^{b}	17.57 ± 1.39^{c}	16.07 ± 1.27^{ac}	17.13 ± 0.84^{ac}	< 0.0001
AI	4.02 ± 0.92	6.11 ± 1.48	5.35 ± 2.64	5.52 ± 1.29	4.43 ± 1.27	0.2905
LDL/HDL	2.78 ± 0.78	4.52 ± 1.13	3.88± 2.07	4.24 ± 1.11	3.20 ± 1.10	0.2296
Body weight	234.16 ± 7.36^{ab}	245 ± 8.94^{b}	229.16 ± 8.01^{a}	240.00 ± 6.12^{ab}	234.16 ± 3.76^{ab}	0.0088
42 days						
HDL-C	14.33 ± 2.08	16.33 ± 1.26	13.33 ± 4.16	15.50 ± 3.54	16.33 ± 1.53	0.4881
TGA	93.37 ± 6.70^{a}	260.20 ± 14.32^{b}	119.43 ± 3.00^{a}	122.70 ± 10.04^{a}	108.73 ± 10.24^{a}	< 0.0001
TC	59.13 ± 1.45^{a}	173.97 ± 8.86^{b}	66.50 ± 0.44^{a}	62.75 ± 1.34^{a}	65.63 ± 3.30^{a}	< 0.0001
LDL-C	26.13 ± 2.04^{a}	110.37 ± 3.82^{b}	29.28 ± 3.43^{a}	21.71 ± 0.18^{a}	27.55 ± 6.24^{a}	< 0.0001
VLDL-C	18.67 ± 1.34^{a}	52.04 ± 2.82^{b}	23.89 ± 0.60^{a}	24.54 ± 2.01^{a}	21.75 ± 2.05^{a}	< 0.0001
AI	3.18 ± 0.57^{a}	9.67 ± 0.33^{b}	4.29 ± 1.49^{a}	2.88± 0.75^{a}	3.05 ± 0.48^{a}	< 0.0001
LDL/HDL	1.87 ± 0.44^{a}	6.82 ± 0.64^{b}	2.38 ± 0.91^{a}	1.35 ± 0.30^{a}	1.64 ± 0.48^{a}	< 0.0001
Body weight	268.33 ± 5.16^{a}	314.16 ± 5.84^{b}	295.83 ± 9.70^{c}	306.00 ± 6.51^{bc}	300.83 ± 7.35^{c}	< 0.0001

Values are represented as mean ± SD., n=6. Means with different letters in the same row are significantly different (P <0.05), checked by Tukey's multiple comparison test, GraphPad prism, ver. 5.0

Abbreviations: ND: Normal diet; HFD: High fat diet Statin: Atrovastatin (Macleod); ARDMC1: *S. cerevisiae* ARDMC1*;* Sb: *S. boulardii*

7.7. Bibliography

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