Pharmacological Study

Influence of intrinsic microbes on phytochemical changes and antioxidant activity of the Ayurvedic fermented medicines: *Balarishta* and *Chandanasava*

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Abstract

Background: *Balarishta* and *Chandanasava* are polyherbal-fermented medicines of Ayurveda. **Objective:** Investigation of native microbes, understanding phytochemical changes and antioxidant activities in these medicines. **Methods:** Microbial populations were enumerated using selective media and standard plating methods. Yeast and bacteria were identified using classical and molecular methods. Qualitative phytochemical and gas chromatography-mass spectrometry (GC-MS) analyses were carried out. *In vitro* antioxidant assays were performed with different assay systems. **Results:** *Balarishta* and *Chandanasava* possess two yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) and six bacteria that are species of *Bacillus*, *Paenibacillus*, and *Brevibacillus*. These microbes identified biochemically were authenticated with 16S and 18S rDNA sequence analysis and NCBI accession numbers. GC-MS analysis indicated that several compounds disappear as a result of fermentation while many are retained. The presence of new phytochemical compounds in the final stages of fermentation could be ascribed from the parent molecules that either disappeared or retained during fermentation. It suggests the biotransformation of phytochemicals by the mediation of intrinsic microbes. These medicines possess antioxidant activities by the presence of phytochemicals such as phenolics, flavonoids, tannins and phytosterols, wherein bacteria also contribute. **Conclusion:** The role of native microbial consortium in fermentation, biotransformation and antioxidant activity of these *Arishta* and *Asava* is demonstrated.

Keywords: 16S and 18S rDNA analysis, antioxidant activity, biotransformation, gas chromatography-mass spectrometry analysis, traditional knowledge

Introduction

Ayurvedic pharmacopeia comprises various medicines including fermented traditional medicines (FTM), namely, *Arishta* (fermented decoctions) and *Asava* (fermented infusions). They are polyherbal preparations fermented by self-generated/intrinsic microbes. They are moderately alcoholic and prepared using herbal juices or their decoctions to undergo fermentation by the addition of sugar, jaggery or honey.[1] Conventionally, *Arishta* and *Asava* are considered as unique and valuable therapeutics[2] because of their better keeping quality, enhanced therapeutic properties, improvement in the extraction of drug molecules from the herbs and effectiveness of drug delivery in the body. There are at least 44 *Arishta* and 45 *Asava* preparations whose composition and medicinal properties were documented earlier.[1-3] Among them, *Balarishta* and *Chandanasava* are commonly used ones.

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Balarishta is recommended for paralysis, nervous disorders, gastric problems, autoimmune diseases and rheumatism. It contains 11 herbal ingredients. *Chandanasava* contains 24 herbal ingredients and is recommended for treating ailments such as gastric problems, urinary disorders, spermatorrhea, gonorrhea, autoimmune diseases and as diuretic, appetizer and to provide cooling effect in the body.[1]

There are few reports on the isolation of microbes from this fermentation system. For example, *Saccharomyces* from *Amritarishta*,[4] yeasts, *Aspergillus* and *Bacillus* species

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from *Dashamularishta*,[5] *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* from *Nimbarishta*.[6] As in other medicaments of Ayurveda, investigation on the phytochemical composition of *Arishta* and *Asava* are also scanty and earlier reports indicate only the presence or absence of certain preliminary groups of phytochemicals.[7-15] There are also few reports on the specific phytochemical components in the group of phenolics, stilbenes, glucosides and non-volatile compounds.[16,17] The situation in *Arishta/Asava* could be complex as microbial fermentation is involved and every feasibility of interaction of the herbal-oriented phytochemicals with the microbial catalyst exists. There are also few reports on the possibility of biotransformation of phytochemicals-phenolics biotransformation in *Arjunarishta* and *Abhayarishta*[16,18] and glucoside biotransformation in *Jirakadyarishta*.[19] It is thus evident that microbiological studies in Ayurvedic *Arishta* and *Asava* are in the nascent stage. The range of microbial catalysts involved in the entire *Arishta* and *Asava* fermentations and their possible succession in different stages need systematic investigation. The analysis of sequence-based evolutionary relationship among the closely related species of 18S rDNA genes of yeasts and 16S rDNA genes of bacteria can be helpful to ascertain their identity.[20] Recently, the antioxidant activity of *Balarishta*,[8,21,22] *Saraswatarishta*,[12] *Ashwagandharishta*[23] and *Kumaryasava*[24] were documented. Antioxidants have numerous pharmaceutical and therapeutic roles to play. Formation of free radicals is the causative factor for several diseases and quenching them by antioxidants leads to health.[25] However, synthetic antioxidants cause side effects and search for natural antioxidants that lack toxicity is the need of the hour.[25] Hence, this work is to evaluate the microbiota emerged during fermentation and their contribution to phytochemical changes and antioxidant property of *Balarishta* and *Chandanasava*.

Materials and Methods

Chemicals and reagents

The chemicals for phytochemical analysis, antioxidant assays and standards were purchased from Himedia (Mumbai, India), Sisco Research Lab (Mumbai, India) and Sigma–Aldrich (St. Louis, Missouri, USA). Chemicals, reagents and solvents used were of analytical grade.

Preparation of *Balarishta* and *Chandanasava*

The Ayurvedic samples were prepared from the manufacturing unit of M/S. Ashtanga Ayurvedics (P) Ltd, Tiruchirappalli, Tamil Nadu, India [Table 1]. The herbal ingredients were sourced directly from this Ayurvedic firm.

Balarishta is made with decoctions of herbs in boiling water, while *Chandanasava* is prepared directly using fresh herbal juices.[1] In the preparation of *Balarishta*, decoction of drug is prepared and placed in an earthen fermentation vessel. Jaggery is dissolved, boiled and added into the fermentation vessel. Fermentation of worts of both the formulation is traditionally brought about by the addition of flowers of the plant, *Woodfordia fruticosa* (L.) kurz. Then, the earthen lid edges are closed with clay smeared cloth. Constant temperature (28°C) is maintained during fermentation. The fermented fluid is finally filtered and used. For *Chandanasava* preparation, the required quantity of water to which jaggery and sugar are added, boiled and cooled. This is poured into the fermentation vessel in that the fine powdered herbal drugs are added. Further processing is performed as in *Balarishta.*

Microbiological evaluation

Enumeration of microorganisms

The samples were decimally diluted (10^{-1}, 10^{-2}-10^{-5}) using sterile 0.85% saline water in 1: 9 ratio. One milliliter of each diluted sample was pour plated using actidione-incorporated nutrient agar (for heterotrophic bacteria),[26] chloramphenicol-incorporated yeast extract-malt extract agar (for yeasts)[27] and chloramphenicol-incorporated dichloron rose bengal agar (for fungi).[28] The total heterotrophic bacterial population (grown at 37°C), yeast (grown at 28°C) and fungal (grown at 30°C) population were enumerated after 42-72 h of growth. Discrete microbial colonies were isolated and purified for further identification.

Biochemical and molecular identification of yeasts using 18S rDNA sequencing

The yeasts were identified using classical biochemical tests such as fermentation of carbohydrates (glucose, sucrose, melibiose, lactose, raffinose, maltose and cellobiose), growth in various carbohydrates and temperatures, urea hydrolysis and reproduction.[27] For 18S rDNA, genomic DNA was isolated with Fungal Genomic DNA Isolation Kit (RKT13, Chromous Biotech, Bengaluru) using pure cultures and amplified internal transcribed spacer (ITS) region of the 18S rDNA genes using universal primers ITS1 (5'-ACCCGCTGAACTTAAGC-3') and ITS2 (5'-TACTACCACCAAGATCT-3') by polymerase chain reaction (PCR).[29,35] The PCR was carried out by denaturing DNA initially at 94°C for 5 min followed by 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 10 min). The presence or absence of PCR products was determined electrophoretically on 1% (w/v) agarose gel with ethidium bromide staining. The PCR products were purified using a Gel Extraction Kit (Qiagen, California, USA) and subcloned into pGEMT-Easy vector (Promega, Wisconsin, USA). The selected clone was subjected to sequencing of 18S rDNA gene fragment with universal primer SP6 (ATTTAGGTGACACTATAGAAGNG) and T7 (TAATACGACTCACTATAGGGAGA) using ABI prism 3130 sequencer (Perkin Elmer, California, USA). The sequence data were aligned with nucleotide PSI-BLAST[30] to identify the closely related organisms and to recognize the origin and evolution among them. Pair-wise and multiple sequence alignment were done by CLUSTAL W.[31] The construction of phylogenetic tree and computing the pair-wise genetic diversity for the yeast isolates was performed based

Table 1: Composition of *Balarishta* and *Chandanasava*

Sanskrit name	Botanical name	Part used	Quantity
	Balarishta		
Bala	*Sida rhombifolia* L.	Root	11 kg
Ashwagandha	*Withania somnifera* (L.) Dunal	Root	11 kg
Guda	Jaggery	-	75 kg
Dhataki	*Woodfordia fruticosa* Kurz.	Flower	700 g
Jivanti	*Holostemma ada-kodien* Schult.	Root	360 g
Eranda	*Ricinus communis* L.	Bark	360 g
Rasna	*Alpinia galanga* Willd.	Root	180 g
Ela	*Elettaria cardamomum* Maton	Fruit	180 g
Prasarini	*Merremia tridentata* (L.) Hallier f.	Root	180 g
Lavanga	*Syzygium aromaticum* (L.) Merr. and L.M.Perry	Flower	180 g
Sugandhimula	*Vetiveria zizanioides* Stapf	Root	180 g
Gokshura	*Tribulus terrestris* L.	Fruit	180 g
-	Water	-	360 L
	Chandanasava		
Chandana	*Santalum album* L.	Wood	120 g
Hribera	*Plectranthus vettiveroides* (Jacob) N.P.Singh and B.D.Sharma	Root	120 g
Musta	*Cyperus rotundus* L.	Tuber	120 g
Kashmari	*Gmelina arborea* Roxb.	Wood	120 g
Indivara	*Monochoria vaginalis* C. Presl	Tuber	120 g
Priyangu	*Callicarpa macrophylla* Vahl.	Flower	120 g
Padmaka	*Prunus cerasoides* D. Don	Wood	120 g
Lodhra	*Symplocos cochinchinensis* S.Moore	Bark	120 g
Manjistha	*Rubia cordifolia* L.	Tuber	120 g
Raktachandana	*Pterocarpus santalinus* L.f.	Wood	120 g
Patha	*Cyclea peltata* (Lam.) Hook.f. and Thomson	Tuber	120 g
Bhunimba	*Andrographis paniculata* Nees	Whole plant	120 g
Vata	*Ficus benghalensis* L.	Bark	120 g
Ashwattha	*Ficus religiosa* L.	Bark	120 g
Shati	*Kaempferia galanga* L.	Tuber	120 g
Parpata	*Hedyotis corymbosa* (L.) Lam.	Whole plant	120 g
Madhuka	*Glycyrrhiza glabra* L.	Rhizome	120 g
Rasna	*Alpinia galanga* Wid.	Tuber	120 g
Patola	*Trichosanthes lobata* Roxb.	Stem	120 g
Kanchanara	*Bauhinia variegata* L.	Bark	120 g
Amra	*Mangifera indica* L.	Bark	120 g
Mocharasa	*Bombax ceiba* L.	Gum	120 g
Mridvika	*Vitis vinifera* L.	Fruit	2400 g
Guda	Jaggery	-	6 kg
Dhataki	*Woodfordia fruticosa* Kurz.	Flower	1800 g
-	Sugar	-	9 kg
-	Water	-	70 L

on sequence similarities using molecular evolutionary genetics analysis (MEGA) Version 6.0 software[32] employing neighbor joining[33] method of Kimura two-parameter evolutionary model with 1000 bootstrap replicates.[34]

Biochemical and molecular identification of bacteria using 16S rDNA sequencing

The bacterial cultures were identified by biochemical tests (Bergey's Manual of Systematic Bacteriology, 1984) such as ram staining, endospore staining, motility, catalase test, fermentation of carbohydrate, indole test, the hydrolysis of starch, casein and gelatin, Voges-Proskauer test, citrate utilization and nitrate reduction.[35] For 16S rDNA, genomic DNA was isolated from the pure culture[29] and 16S rDNA was amplified using universal primers (FD1-'-GAGTTTGATCCTGGCTCAG-3 ' and RD1-5'-AAGGAGGTGATCCAGCC-3'). PCR was carried out by denaturing DNA initially at 94°C for 5 min followed by 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 2 min). The PCR amplified products were electrophoresed on 1% agarose gel. The PCR products were purified using a Gel Extraction

Kit (Qiagen, California, USA) and subcloned into pGEM-T Easy vector (Promega, Wisconsin, USA). The selected clone was subjected to sequencing of 16S rRNA gene fragment with universal primer SP6 (ATTTAGGTGACACTATAGAAGNG) and T7 (TAATACGACTCACTATAGGGAGA) using ABI prism 3130 sequencer (Perkin Elmer, California, USA).[36] Phylogenetic analysis of the sequence data (PSI-BLAST, CLUSTAL W and MEGA) was performed as in the case of yeasts.

Phytochemical evaluation

Qualitative analysis of phytochemicals

The preliminary phytochemicals were evaluated up to 35 days in 5-day intervals during the course of fermentation.[37] Alkaloids were analyzed using Dragendorff's reagent and saponins by foaming test[38] glycosides were assessed by analyzing the total sugar content of the sample,[39] before and after hydrolysis with concentrated sulfuric acid,[38] tannins using lead acetate reagent, phenolic compounds using ferric chloride reagent, phytosterols using acetic anhydride-sulfuric acid reagent and flavonoids using lead acetate reagent.[40]

Gas chromatography-mass spectrometry analysis of phytochemicals

Arishta and *Asava* samples were concentrated in hot air oven at 80°C for 24-48 h. Fifteen milliliters of this sample was frozen using the deep freezer for 1 day at –20°C and then, the frozen sample was concentrated with the help of vacuum evaporator at –80°C. The freeze-dried sample was dissolved in 10 ml of HPLC grade methanol and gas chromatography-mass spectrometry (GC-MS) (PerkinElmer Clarus 500, Connecticut, USA) analysis was performed.[41] One micro liter of sample (*Balarishta* and *Chandanasava* separately) was injected (split ratio 1:8) into the GC-MS system on a 30-m capillary column with a film thickness of 0.25 µm (30 mm × 0.25 mm id coated with 5% phenyl 95% dimethylpolysiloxane). Helium was the carrier gas with a flow rate of 1 ml/min. The injection port temperature was 280°C. Two types of oven temperature were followed. Initial oven temperature of 50°C at 10°C/min to 150°C at 8°C/min to 280°C (10 min) and 60°C at 8°C/min to 200°C at 10°C/min to 300°C (5 min) (Scan type: full scan mode, Scan range: 40-450 Daltons). The peaks are matched with phytochemistry library: NIST (The National Institute of Standards and Technology) MS search library version 2.0.

Pharmaceutical evaluation

Antioxidant assays

The antioxidant activity of *Balarishta*, *Chandanasava,* and microbial cell-free supernatant (CFS) were measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity,[23,42] hydroxyl radical scavenging,[42,43] hydrogen peroxide scavenging,[23] reducing power,[23,42] metal chelating assay,[43,44] nitric oxide scavenging,[43] superoxide anion[43,44] and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid [ABTS]) radical scavenging assay.[45] (Jasco V-650, Tokyo, Japan).

Statistical analyses

The data were represented as mean ± standard error of mean of three determinations. Statistical analyses were performed using one-way analysis of variance. The IC_{50} values were calculated by linear-regression probit analysis. Results were calculated by employing the statistical software (SPSS for Windows, Version 16.0. Chicago, SPSS Inc.).

Results

Microbiological evaluation

The population of bacteria, yeast, and fungi was investigated during the entire course of fermentation of *Balarishta* [Figure 1a] and *Chandanasava* [Figure 1b]. In both *Chandanasava* and *Balarishta,* filamentous fungi were found only at the beginning stages of fermentation that too at low levels over the surface. They disappeared from the fifth day in *Balarishta* and tenth day in *Chandanasava.* In *Balarishta*, yeasts are the dominant population found throughout the course of fermentation (25,700 × 10^3 CFU/ml). The population was at its peak on the fifth day and subsequently decreased toward the end of fermentation [Figure 1a]. However, in *Chandanasava*, though yeasts were dominant (9500 × 10^3 CFU/ml), they disappeared after twenty fifth day. The peak of yeast population was found on fifth and tenth days. Next to yeasts, the presence of bacteria was also observed in

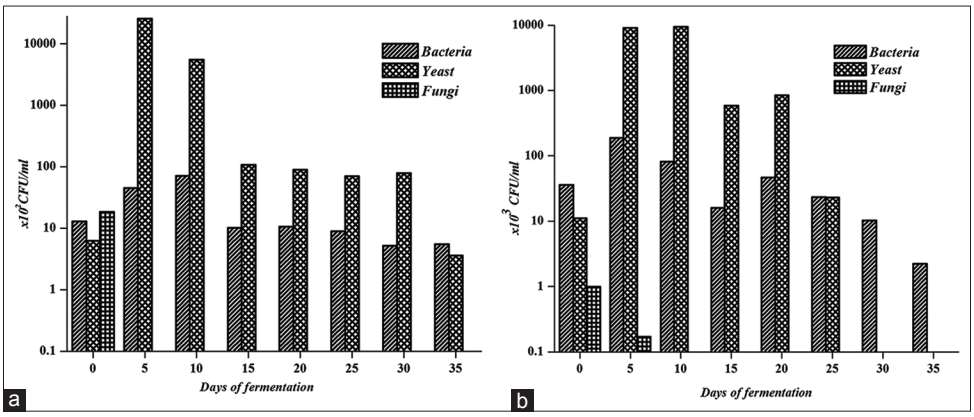


Figure 1: Enumeration of microbial population during the course of fermentation of (a) *Balarishta* and (b) *Chandanasava*

this fermentation process [Figure 1b]. In *Balarishta,* the bacterial population gradually increased up to tenth day and subsequently maintained at steady level (71×10^3 CFU/ml) till the end of fermentation [Figure 1a]. But, in *Chandanasava,* the bacterial population was at its peak on the fifth day (188×10^3 CFU/ml). It decreased, but fluctuated till the end of fermentation [Figure 1b]. Investigation of yeast population indicated the presence of two kinds that are similar in both *Balarishta* and *Chandanasava*. Both organisms have the same colony morphology – creamy white and circular colonies. Microscopic examination revealed the presence of budding yeast and fission yeast.

Identification of budding yeast (BY-03) by morphological and biochemical features [Table 2] revealed as *S. cerevisiae*. Similar identification of fission yeast (BY-10) revealed as *S. pombe* [Table 1]. The bacteria present in the *Balarishta* fermentation was investigated morphologically and biochemically [Table 3]. It revealed the presence of six species of *Bacillus* (*Bacillus licheniformis, Bacillus macerans*, *Bacillus pumilus*, *Bacillus subtilis* and *Bacillus circulans*). Similarly, in *Chandanasava*, six species of *Bacillus* (*B. licheniformis*, *B. subtilis*, *B. polymyxa*, *B. coagulans*, *B. circulans* and *B. mycoides*) were present [Table 3].

Occurrence of bacterial flora in the suspension of *Balarishta* and *Chandanasava* during the entire course of fermentation was recorded. In *Balarishta, B. pumilus* was constantly present, while other five species had a sporadically present during the full course of fermentation. *B. licheniformis* was noticed in the fermentation mash on 0, 15, 20, 25 and 35 days. *B. brevis* was found on 0, 10 and 20 days. *B. circulans* was found on 5, 20 and 30 days. *B. subtilis* was observed on 5, 10 and 30 days. Finally, *B. macerans* was found on 0, 5 and 10 days. In *Chandanasava*, *B. licheniformis* and *B. subtilis* were constantly present while another four species showed the sporadic presence during the full course of fermentation. *B. polymyxa* and *B. coagulans* were found only at the initial day of fermentation and absent subsequently. *B. mycoides* was noticed only on twentieth day, while *B. circulans* was found only on 15, 20 and 30 days of fermentation.

Table 2: Biochemical characterization leading to the identification of yeasts involved in the fermentation of *Balarishta* and *Chandanasava*

Parameters	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*
Glucose fermentation	+	+
Sucrose fermentation	+	+
Melibiose fermentation	+	-
Lactose fermentation	-	-
Growth in glucose	+	+
Growth in sucrose	+	+
Growth in raffinose	+	+
Growth in maltose	+	+
Growth in melibiose	+	-
Growth in lactose	-	-
Growth in cellobiose	-	-
Growth at 25°C	+	+
Growth at 30°C	+	+
Growth at 35°C	+	+
Growth at 37°C	+	+
Urea hydrolysis	-	+
Growth in 50% glucose	+	+
Growth in 10%NaCl	+	+
Growth in 16% NaCl	-	-
Reproduction	Budding	Fission

+: Positive for the test, -: Negative for the test

Budding yeast (BY-03) was identified by 18S rDNA as *S. cerevisiae* (Accession number: KJ562355) [Table 4]. Similarly, fission yeast (BY-10) was identified as *S. pombe* (Accession number: KJ562356) [Table 4]. The Homologs template sequences were retrieved from NCBI-BLAST based on the similarity analysis from our target isolates of 12 bacteria and 2 yeasts [Table 3]. Identification of 12 bacteria by 16S rDNA revealed some differences from the classical biochemical tests based approaches, [Table 4] wherein the organisms fit into species of *Bacillus*, *Paenibacillu,* or *Brevibacillus*. Using neighbor-joining algorithm, the phylogenetic tree was constructed with 1000 bootstrap replication [Figure 2]. The phylogenetic tree was grouped into two major clusters. Cluster one belongs to *S. cerevisiae* and cluster two belongs to *S. pombe*. The sequence-based evolutionary relationship among the strains of *S. cerevisiae* shows no diversity occurrence at the species level. Our target sequence *S. cerevisiae* BY-03 is closely related with *S. cerevisiae* Sc20. Whereas, *S. pombe* BY-10 are closely related with the genera *Schizosaccharomyces* UFLA [Figure 3]. From this result, the closely related species were placed as a reference model for the target species.

Phytochemical evaluation

Preliminary phytochemical changes

Qualitative analysis of phytochemicals during the process of fermentation of *Balarishta* indicated some major changes, though alkaloids, tannins, phenolic compounds and glycosides were present during the entire course of fermentation. Flavonoids and saponins were found from fifth-day onward, whereas phytosterol from the tenth-day onward. Similar

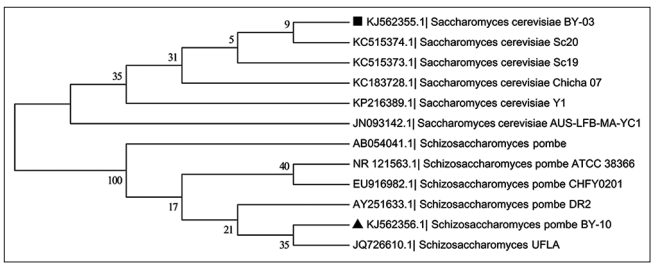


Figure 2: Phylogenetic tree based on 16S rDNA genes of the 12 bacterial isolates. The numbers at nodes are percentages indicating the levels of bootstrap support, based on neighbor joining method. ♦ Represents bacterial isolates from *Balarishta.* ● Represents bacterial isolates from *Chandanasava*

Table 3: Biochemical characterization leading to the identification of bacteria involved in the fermentation of *Balarishta* and *Chandanasava*

Parameters	*Balarishta*								*Chandanasava*			
	B. licheniformis	*B. subtilis*	*B. circulans*	*B. macerans*	*B. pumilus*	*B. brevis*	*B. licheniformis*	*B. subtilis*	*B. circulans*	*B. coagulans*	*B. polymyxa*	*B. mycoides*
Gram's staining	+	+	+	+	+	+	+	+	+	+	+	+
Spore staining	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	-	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	-	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	-	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	-	-
VP test	+	+	-	-	+	-	+	+	-	+	+	+
pH of VP	<6	<6	<6	<6	<6	>7	<6	<6	<6	<6	<6	<6
Utilization of citrate	+	+	+	+	+	+	+	+	+	+	-	+
Nitrate reduction	+	+	+	+	-	+	+	+	+	+	+	+
Anaerobic growth	+	-	+	+	-	-	+	-	+	+	+	+
Acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+
Acid from mannitol	+	+	+	+	+	+	+	+	+	+	+	-
Acid from xylose	+	+	+	+	+	-	+	+	+	+	+	-
Gas from glucose	-	-	-	+	-	-	-	-	-	-	+	-

+: Positive for the test, -: Negative for the test, *B. licheniformis*: *Bacillus licheniformis*, *B. subtilis*: *Bacillus. subtilis*, *B. circulans*: *Bacillus circulans*, *B. macerans*: *Bacillus macerans*, *B. pumilus*: *Bacillus pumilus*, *B. brevis*: *Bacillus brevis*

phytochemical changes in *Chandanasava* showed the presence of alkaloids, tannins, phenolic compounds, saponins and glycosides in the entire course of fermentation. Further, phytosterols and flavonoids were found from fifth day onward.

Specific phytochemical changes

To ascertain the nature of phytochemicals, GC-MS analysis was performed using two types of oven temperature programs, both at the initial and final stages of fermentation in *Balarishta* and *Chandanasava.* The compounds identified at the start of fermentation and at the end of fermentation are listed out, respectively, for *Balarishta* and *Chandanasava*. Analysis of these results indicated that certain compounds are retained during the entire course of fermentation while many disappear. Similarly, formation of new compounds can be traced. Based on this GC-MS analysis, there are 19 compounds retained during the entire course of fermentation of *Balarishta*. Further, 18 compounds are known to disappear and 23 compounds are newly formed as a result of fermentation [Table 5]. In the case of *Chandanasava*, 14 compounds are retained during the entire course of fermentation. Further, 30 compounds are known to disappear and 32 compounds are newly formed as a result of fermentation [Table 6].

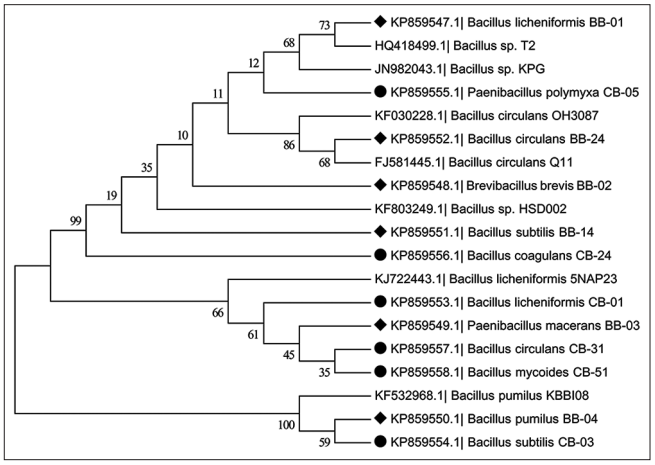


Figure 3: Phylogenetic tree based on 18S rDNA genes of the two yeast isolates (BY-03 and BY-10). The numbers at nodes are percentages indicating the levels of bootstrap support, based on neighbor joining method

Table 4: Similarity search analysis of rRNA gene sequences of bacteria (16S) and yeasts (18S) isolated from *Balarishta* and *Chandanasava* using NCBI-BLAST

Target		Template	
Identification	**Accession**	**Homology strain**	**Identity (%)**
		Bacteria	
Bacillus licheniformis BB-01	KP859547	*Bacillus* spp. KPG	91
Brevibacillus brevis BB-02	KP859548	*Brevibacillus brevis* strain ZFJ-2	100
Paenibacillus macreans BB-03	KP859549	*Bacillus* spp. hb7	99
Bacillus pumilus BB-04	KP859550	*Bacillus pumilus* strain KBBI08	98
Bacillus subtilis BB-14	KP859551	*Bacillus* spp. HSD002	99
Bacillus circulans BB-24	KP859552	*Bacillus circulans* strain Q11	96
Bacillus licheniformis CB-01	KP859553	*Bacillus licheniformis* strain 5NAP23	98
Bacillus subtilis CB-03	KP859554	*Bacillus subtilis* strain ZJ-1	93
Paenibacillus polymyxa CB-05	KP859555	*Bacillus* spp. A-BT-nw	99
Bacillus coagulans CB-24	KP859556	*Bacillus* spp. I7 (2011)	99
Bacillus circulans CB-31	KP859557	*Bacillus* spp. D12 (2010)	99
Bacillus mycoides CB-51	KP859558	*Bacillus* spp. AY8	98
		Yeasts	
Saccharomyces cerevisiae BY-03	KJ562355	*Saccharomyces cerevisiae* isolate AUS-LFB-MA-YC1	97
Schizosaccharomyces pombe BY-10	KJ562356	*Schizosaccharomyces japonicus*	98

NCBI-BLAST: National Center for Biotechnology Information-Basic Local Alignment Search Tool

Table 5: Phytochemicals that are retained, disappeared and newly formed as a result of fermentation of *Balarishta*

Retained compounds	Disappeared compounds	Newly formed compounds
2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one	1,2,3-Propanetriol, monoacetate	1,2-Epoxy-3-propyl acetate
2,5-Dimethyl-4-hydroxy-3 (2H)-furanone	1,2-Benzenedicarboxylic acid, butyl octyl ester	1H-Imidazole-4-ethanamine, N,5-dimethyl-
2-Furancarboxaldehyde, 5-(hydroxymethyl)-	1,3-Dioxane, 2,4-dimethyl-	2 (3H)-Furanone, dihydro-4-hydroxy-
2-Furancarboxaldehyde, 5-methyl-	1b, 4a-Epoxy-2H cyclopenta[3,4]cyclopropa[8,9] cycloundec [1,2- b] oxiren-5 (6H)-one, 7 (acetyloxy) decahydro-2,9,10-trihydroxy- 3,6,8,8,10a-pentamethyl-	2,2'-Bioxirane, (R*, R*)-(ñ)-
2-Furanmethanol	2-Cyclopenten-1-one, 2-hydroxy-3-methyl-	2,3-Butanediol
2H-Pyran-2,6 (3H)-dione	3-Butyl-4-nitro-pent-4-enoic acid, methyl ester	2-Deoxy-D-galactose
2-Propanone, 1,3-dihydroxy-	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	2-Formyl-9-[α -d-ribofuranosyl] hypoxanthine
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	5-Acetoxymethyl-2-furaldehyde	(s) 2-Hydroxypropanoic acid
6-Oxa-bicyclo[3.1.0]hexan-3-one	7-Methyl-Z-tetradecen-1-ol acetate	2-Propanone, 1-hydroxy-
α -D-Glucopyranose, 4-O- α -D-galactopyranosyl-	d-Mannose	2-Propanamine, N-methyl-N-nitroso-
α -D-Glucopyranoside, O- α -D-glucopyranosyl-(1.fwdarw. 3)- α -D-fructofuranosyl	D-Streptamine, O-2-amino-2-deoxy- α -D-glucopyranosyl-(14)-O-[O- 2,6-diamino-2,6-dideoxy- α -L-idopyranosyl-(13)- α -D- ribofuranosyl-(15)]-2-deoxy-	2-Propenoic acid, ethenyl ester
Desulfosinigrin	Ethanone, 1-(2-furanyl)-	4-Aminoisoxazolidin-3-one
D-Glucose, 4-O- α -D-glucopyranosyl-	Ethyl iso-allocholate	6-Acetyl- α -d-mannose
d-Glycero-d-ido-heptose	N-Methoxy-1-ribofuranosyl-4-imidazolecarboxylic amide	Acetic acid, 1-methylethyl ester
DL-Arabinose	N-Nitroso-2,4,4-trimethyloxazolidine	Carbonocyanidic acid, ethyl ester
Furfural	Pentanol, 5-amino-	Dibutyl phthalate
Tetrahydropyrrole-3-amino-2,5-dione	Phthalic acid, butyl undecyl ester	Glycerin
Uric acid	Propanoic acid, 2-oxo-, methyl ester	Imidazole, 2-amino-5-[(2-carboxy) vinyl]-
Hydrazinecarboxamide, 2-(2-methylcyclohexylidene)-		Isosorbide Dinitrate
		Lupeol
		Oxirane, 2,3-dimethyl-, trans-
		Pentylamine, N-isobutyl-N-nitroso-
		R-(-)-1,2-propanediol

Pharmaceutical evaluation

Antioxidant activity

Both *Balarishta* and *Chandansava* exhibited considerable antioxidant activities. It was assayed in terms of scavenging of free radicals (DPPH, HRSA, hydrogen peroxide, ABTS, nitric oxide and superoxide anion), inhibition of free radical generation by metal chelation [Table 7] and assessing the reducing power [Figure 4]. In DPPH assay, IC_{50} value of *Balarishta* (250.48 µl/ml) and *Chandanasava* (442.99 µl/ml) were higher when compared to the standard, L-ascorbic acid (210.31 µg/ml) [Table 7]. It indicated that *Chandanasava* has low DPPH scavenging activity compared to *Balarishta.*

Table 6: Phytochemicals that are retained, disappeared and newly formed as a result of fermentation of *Chandanasava*

Retained compounds	Disappeared compounds	Newly formed compounds
1,2,3-Benzenetriol	1,2,3-Propanetriol, monoacetate	1,2-Benzenediol
2,3-Butanediol, [S-(R*, R*)]-	1,2,4-Benzenetriol	1,2-Cyclopentanedione
2,5-Furandione, dihydro-3-methylene-	1,3-Dioxol-2-one, 4,5-dimethyl-	1,4-Anhydro-d-galactitol
2-Furancarboxaldehyde, 5-methyl-	1,4-Dioxin, 2,3-dihydro-	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one
2-Furanmethanol	1,6-Anhydro- α -D-glucopyranose (levoglucosan)	2,4-Hexanediol, 5-methyl-3-nitro-
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	1-Isobutyl-7,7-dimethyl-octahydro-isobenzofuran- 3a-ol	2H-Pyran-2,6 (3H)-dione
4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	2,5-Dimethyl-4-hydroxy-3 (2H)-furanone	2-Methoxy-4-vinylphenol
Benzeneethanol, 4-hydroxy-	2,5-Furandicarboxaldehyde	3,4Dehydro-dl-proline
Diglycerol	2,5-Methylene-d, l-rhamnitol	3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-
Glycerin	2-acetonyl-9-[3-deoxy-.beta.-d-ribouranosyl] hypoxanthine	3-O-Methyl-d-glucose
Lactic acid	2-Butanone, 4-methoxy-	4-(2-Hydroxyethyl) phenol
Lactose	2-Cyclopenten-1-one, 2-hydroxy-	α-Copaen-11-ol
Squalene	2-Cyclopentene-1,4-dione	α -d-Lyxofuranoside, methyl
2-Furancarboxaldehyde, 5-(hydroxymethyl)-	3-Deoxy-d-mannoic lactone	Trans-squalene
	5-Hydrxoymethylfurfural	α -Sitosterol
	5-Oxotetrahydrofuran-2-carboxylic acid, ethyl ester	Benzofuran, 2,3-dihydro-
	6-Acetyl-α -d-mannose	Carissanol
	Acetate, [4-hydroxy-4-(1-methylethyl)-5-methyl-2- hexynyl] ester	Cholesta-4,6-dien-3-ol, (3α)-
	α -D-Glucopyranoside, O-α -D-glucopyranosyl -(1.fwdarw. 3)- α -D- fructofuranosyl	Dianhydromannitol
	Andrographolide	d-Mannitol, 1,4-anhydro-
	d-Glycero-d-ido-heptose	d-Mannose
	Ether, 3-butenyl pentyl	Ergost-5-en-3-ol, acetate, (3α,24R)-
	Furfural	Ethyl α -d-glucopyranoside
	Glucosamine, N-acetyl-N-benzoyl-	Ethyl hydrogen succinate
	Glycero-d-ido-heptose	Isosorbide
	Lactic acid, methyl ester	L-xylose
	L-Glucose	n-Hexadecanoic acid
	Maltol	Phenol, 2,6-dimethoxy-
	N-Nitroso-2,4,4-trimethyloxazolidine	Phenylethyl Alcohol
	Tridecanoic acid, methyl ester	Stigmastan-3,5-diene
		Stigmastan-6,22-dien, 3,5-dedihydro-
		Succinic anhydride

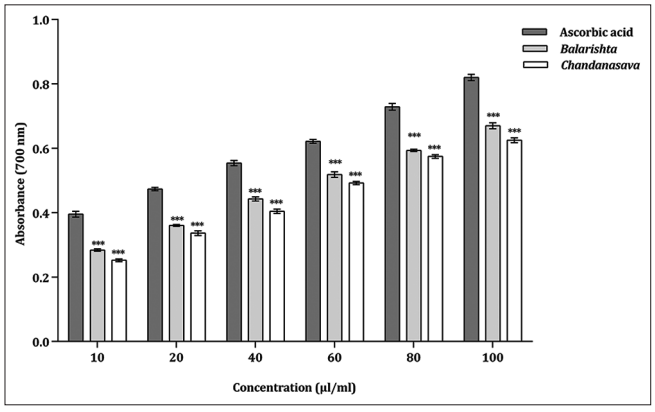


Figure 4: Reducing power of *Balarishta* and *Chandanasava*. Values are represented as mean ± standard error of mean ($n = 3$). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus control. Ascorbic acid is the standard (µg/ml)

Similar trend was observed in hydroxyl, nitric oxide and superoxide anion radicals scavenging assays [Table 7]. However, in hydrogen peroxide scavenging assay, *Chandanasava* (56.57 µl/ml) performed better than *Balarishta* (69.22 µl/ml), yet their performance was less than the standard L-ascorbic acid (30.09 µg/ml). Similar trend was observed in ABTS scavenging and metal chelating assay also [Table 7]. However, the efficacy of *Chandanasava* in ABTS scavenging assay was comparable to the standard. In reducing power assay, both *Balarishta* and *Chandasava* were less compared to the standard. Among them, *Balarishta* exhibited better reducing power ability than *Chandasava* [Figure 4].

It is important to note that CFSs of bacteria obtained from *Balarishta* and *Chandansava* also exhibited antioxidant property. However, the activity was less when compared to the standards in all the assays [Table 8]. In DPPH assay, among the bacterial isolates of *Balarishta*, BB-14 (62.6%) showed highest free radical scavenging ability. Similar trend was observed in hydroxyl radical scavenging assay also. In ABTS assay, BB-04 (75.56%) performed better in scavenging. In nitric oxide and metal chelating assays, BB-01 was better. Similarly, BB-24 was better in superoxide anion and reducing power [Table 8].

Table 7: Antioxidant activity of *Balarishta* and *Chandanasava*

Assays	IC_{50} value		
	Standard (μg/ml)	*Balarishta* (μl/ml)	*Chandanasava* (μl/ml)
Scavenging of free radicals			
DPPH	210.31 (ascorbic acid)	250.48	442.99
Hydroxyl radical	30.31 (ascorbic acid)	51.76	67.62
Hydrogen peroxide	30.09 (ascorbic acid)	69.22	56.57
ABTS	28.50 (BHT)	45.55	26.35
Nitric oxide	29.00 (ascorbic acid)	36.30	52.95
Superoxide anion	25.08 (ascorbic acid)	33.45	36.74
Inhibition of free radical generation			
Metal chelating	22.75 (citric acid)	63.48	54.91

IC_{50} value was determined to be the effective concentration at which the particular free radical was scavenged by 50%. The IC_{50} value was obtained by linear-regression probit analysis. DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

Table 8: Antioxidant activity of cell-free supernatant of bacteria isolated from *Balarishta* and *Chandanasava*

Samples	Antioxidants assay (%)						
	Scavenging of free radicals					Inhibition of free radical generation	H^+ - donating ability
	DPPH	Hydroxyl radical	ABTS	Nitric oxide	Super oxide anion	Metal chelating	Reducing power
Standard	78.47±0.235 (AA 100 μg/ml)	79.62±0.02 (AA 100 μg/ml)	77.15±0.016 (BHT 100μg/ml)	79.58±0.662 (AA 100 μg/ml)	95.01±0.047 (AA 100 μg/ml)	87.43±0.032 (CA 100 μg/ml)	81.61±0.094 (AA 100 μg/ml)
Bacterial isolates	Concentration of culture filtrate						
	1 ml	1 ml	1 ml	1 ml	1 ml	0.5 ml	0.5 ml
Bacillus licheniformis BB-01	52.69±0.399	54.73±0.335	54.41±0.043	62.66±0.649	73.91±0.095	78.85±0.058	34.03±0.184
Brevibacillus brevis BB-02	46.03±0.045	56.74±0.504	63.67±0.344	61.67±0.867	74.68±0.334	71.46±0.596	38.30±0.040
Paenibacillus macreans BB-03	61.78±0.149	53.54±0.804	72.82±1.051	60.12±0.708	76.33±0.172	67.58±0.686	40.60±0.126
Bacillus pumilus BB-04	48.04±0.341	55.35±0.611	75.56±0.427	61.76±0.706	74.98±0.290	58.65±0.749	44.54±0.633
Bacillus subtilis BB-14	62.60±0.269	58.98±0.511	59.62±0.514	61.07±0.298	76.14±0.312	68.04±0.319	39.49±0.228
Bacillus circulans BB-24	52.66±0.325	50.74±0.414	62.47±0.556	61.41±0.891	76.69±1.508	40.94±0.120	44.76±0.938
Bacillus licheniformis CB-01	64.32±0.239	51.88±0.629	42.32±0.810	59.20±0.458	68.70±0.312	78.28±0.302	45.32±0.051
Bacillus subtilis CB-03	60.56±0.399	61.34±0.536	58.17±0.504	60.14±0.593	68.78±0.455	79.64±0.890	57.52±0.475
Paenibacillus polymyxa CB-05	57.28±0.260	59.47±0.770	69.51±0.552	60.33±0.267	73.08±0.312	74.24±0.754	43.57±0.088
Bacillus coagulans CB-24	66.86±0.534	52.10±0.715	75.62±0.226	60.60±0.387	70.90±1.576	80.28±0.915	45.10±0.203
Bacillus circulans CB-31	70.03±0.572	54.73±0.511	62.52±0.585	57.75±0.352	73.99±0.252	77.60±0.093	61.26±0.088
Bacillus mycoides CB-51	64.58±0.613	50.92±0.007	69.30±0.269	55.71±0.187	77.96±0.407	78.72±0.137	69.34±0.804

AA: Ascorbic Acid, BHT: Butylated hydroxyl toluene, CA: Citric acid. The presented values are expressed as mean±SEM of three independent experiments. DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), SEM: Standard error of the mean

In *Chandanasava*, scavenging of DPPH radical was better in CB-31 (70.03%). CB-03 showed better hydroxyl radical scavenging ability among the isolates. In ABTS, nitric oxide and metal chelating assays, CB-24 exhibited the highest activity followed by others [Table 8]. CB-51 had higher superoxide anion radical scavenging and H+ donating ability (reducing power).

Discussion

The profile of enumerated microorganisms reveals that yeasts and bacteria are the major players in the fermentation of both *Balarishta* and *Chandanasava*. The disappearance of filamentous fungi at the early stages of fermentation in both cases indicates that they have no role to play in the fermentation process. Moreover, yeasts are well known for their ability of alcohol production. It is possible that either yeasts or bacteria or their combination consortia could be the biocatalysts for the biotransformation of phytochemicals present in both preparations. It is important to note that there are two kinds of yeasts *S. cerevisiae* and *S. pombe* coupled with six different species of bacilli that are present in both *Balarishta* and *Chandanasava.*

In this study, the profile of bacteria changes with the progression of fermentation. Although both yeasts are commonly present, the species of *Bacillus* showed dynamic changes during the course of fermentation. Similarly, in *Kutajarishta*,

microbial composition at initial stages of fermentation was assessed by culture-independent 16S rDNA gene clone library approach.[14] At the initial stage of fermentation, *Lactobacillus* sp., *Acinetobacter* sp., *Alcaligenes* sp. and *Methylobacterium* sp. were recovered but were not detected on the eighth day of fermentation. Initially, microbial diversity increased after 8 days of fermentation with 11 Operational Taxonomic Units (OTUs), which further decreased to 3 OTUs at 30 days of fermentation. *Aeromonas* sp., *Pseudomonas* sp. and *Klebsiella* sp. dominated till the thirtieth day of fermentation.

Herbal materials were indicated as a source of bacterial species.[46] Dried flower buds of *W. fruticosa* (an ingredient) were reported as a source of *Aspergilus* sp. cocci and rod-shaped bacilli[4] as well as *Rhizopus nigricans* and *Aspergillus niger*.[47] Addition of *W. fruticosa* was known to increase the content of alcohol.[48,49] From commercial *Nimbarishta*, *S. cerevisiae* Hansen and *S. pombe* Lindner var. *pombe* were reported to be isolated.[6] *Saccharomyces* species were also isolated and characterized from *Amritarishta*.[4] Two species of yeasts were isolated from *Dashamularishta* along with *Aspergillus* and *Bacillus* species.[5] The phylogenetic analysis supported the identification of ancestral and closely related species of *Saccharomyces, Schizosaccharomyces,* and the different species of *Bacillus*, *Paenibacillus or Brevibacillus* in both *Balarishta* and *Chandanasava.*

Qualitatively, *Balarishta* and *Chandanasava* have various secondary metabolites. Such phytochemical analysis in various *Arishta* and *Asava* indicated the presence of secondary plant metabolites such as phenols, flavonoids, terpenoids, glycosides, steroids, alkaloids, tannins, saponins and various alcohols and related compounds.[7-15] However, certain compounds such as flavonoids, saponins and phytosterols form at a later stage in the course of fermentation as evident in *Balarishta* and *Chandanasava* predicting the role of microorganisms present in.

The plethora of chemicals present in the final product would also have been formed as a result of microbial biotransformation. It is supported by the observation that in *Balarishta*, 18 compounds disappeared as a result of fermentation while 23 compounds are newly formed. In addition, 19 compounds are retained from the beginning up to the final product. In the case of *Chandanasava*, 30 compounds disappeared as a result of fermentation while 32 compounds are newly formed. In addition to this, 14 compounds are retained from the beginning to the final product. Particularly, newly formed chemicals could have been formed from disappearing compounds or from the retained compounds

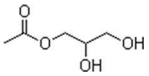
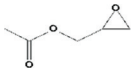
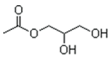
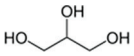
In *jujube Asava* prepared in Japan, hydrolysis of glycosides is known as a result of fermentation (benzyl alcohol from zizybeoside I or II present in jujube).[50] In *Abhayarishta,* the major polyphenolics (Chebulagic and Chebulinic acid) were hydrolyzed to their respective monomers indicating biotransformation and consequently, there was an increase in the amount of chebulic acid, gallic acid, ellagic acid and ethyl gallate after fermentation.[18] In *Jirakadyarishta*, selective hydrolysis of 7-O-glucosides of luteolin and apigenin during fermentation resulted in an increase in the amount of luteolin and apigenin indicating biotransformation.[19] In *Arjunarishta*, the possible hydrolysis of ellagitannins and gallotannins during fermentation, resulting in an increase in the concentration of monomeric phenolics indicating biotransformation.[16]

In our studies also, the possible biotransformation of phytochemicals and their reactions could be chemically assessed. In the case of *Balarishta,* for example, the formation of 2,3-butanediol from oxirane, 2,3-dimethyl-trans is feasible by hydroxylation. Similarly, the formation of three other biotransformed compounds in the final product could be chemically supported from the source compounds [Table 9]. It could be possible by reactions such as removal of water, hydrolysis of ester and reduction of the carbonyl group as warranted in the biotransformation reactions. Similarly, in the case of *Chandanasava*, the possible biotransformation of eight such phytochemicals with their reactions is indicated [Table 10]. Here also, chemical reactions involving removal of the hydroxyl group, ketoenol tautomerism, isomerization, deacetylation, ester hydrolysis etc., could be implicated as the basis for biotransformation reactions. Such reactions could have been mediated by the intrinsic microflora of microorganisms acquired during fermentation.

Antioxidants are molecules or systems that inhibit or quench free radicals or interrupt propagation of the free radicals generated by reactive species.[25] It is extensively reported that several diseases are caused due to the oxidative stress generated by reactive oxygen species (ROS). Endogenous antioxidants are very delicate because the imbalance between overproduction of ROS and less neutralization ability of free radicals which results cellular damage.[51] Researchers reported that synthetic antioxidants are helpful to inhibit the free radicals generation, but it causes side effects leading to several diseases.[25] In this regard, there is a need to search natural antioxidants which are believed to scavenge free radicals and also considered as safe.

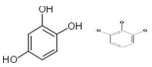
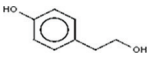
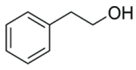
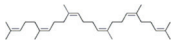
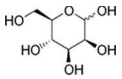
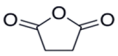
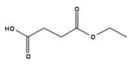
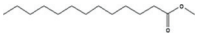
DPPH radical is a stable organic molecule and useful in the investigation of free radical scavenging by antioxidants and hence used primarily in the assay system.[52] Hydroxyl radicals are potent ROS that react with polyunsaturated fatty acid and moieties of cell membrane phospholipids and cause damage to cells.[53] Hydrogen peroxide generates extremely reactive hydroxyl radicals in the presence of transition metal ions such as iron and copper, rapidly cross the cell membranes and pose higher toxicity to cells.[24] ABTS radical scavenging activity is based on the transfer of hydrogen atoms and electrons.[45] Superoxide radicals are most harmful radical because they are the precursors for other major ROS.[45] Nitric oxide radical is generated from L-arginine metabolism by nitric oxide synthases and are toxic to biological tissues. The toxicity is increased, when NO radicals react with superoxide radical to form a highly reactive peroxynitrite

Table 9: Feasible biotransformation reactions in *Balarishta* based on the changes in the profile of compounds upon fermentation as identified by GC-MS analysis

Source compound (retained/ disappeared/newly formed)	Possible reaction(s)	Biotransformed compound present in the final product
 Oxirane, 2,3-dimethyl-, trans-	Hydroxylation →	 2,3-Butanediol
 1,2,3-Propanetriol, monoacetate	Removal of water →	 1,2-Epoxy-3-propyl acetate
 1,2,3-Propanetriol, monoacetate	Hydrolysis of ester →	 + Acetic acid Glycerin
 Propanoic acid, 2-oxo-, methyl ester	Reduction of carbonyl group and hydrolysis of ester →	 (S)-2-Hydroxypropanoic acid

GC-MS: Gas chromatography-mass spectrometry

Table 10: Feasible biotransformation reactions in *Chandanasava* based on the changes in the profile of compounds upon fermentation as identified by GC-MS analysis

Source compound (retained/ disappeared/newly formed)	Possible reaction(s)	Biotransformed compound present in the final product
 1,2,4-Benzenetriol or 1,2,3-Benzenetriol	Removal of hydroxyl group →	 1,2-Benzenediol
 2-Cyclopenten-1-one, 2-hydroxy-	Keto-enol tautomerism → ←	 1,2-Cyclopentanedione
 4-(2-Hydroxyethyl) phenol	Removal of hydroxyl group →	 Phenylethyl Alcohol
 Squalene	Conversion of CIS to trans isomer →	 Trans squalene
6-Acetyl-β-d-mannose	Deacetylation →	 d-Mannose
 Succinic anhydride	Addition of ethanol →	 Ethyl hydrogen succinate
 Tridecanoic acid, methyl ester	Ester hydrolysis →	 n-Hexadecanoic acid
Lactic acid methyl ester	Ester hydrolysis →	 Lactic acid

GC-MS: Gas chromatography-mass spectrometry

anion (ONOO−).[52] Reduction of Fe^{3+} to Fe^{2+} indicates the presence of reductones (antioxidants) which reflects scavenging of free radicals by donating a hydrogen atom.[54] Iron is vital for carrying oxygen, respiration and for enzymatic activity. It can initiate lipid peroxidation by Haber–Weiss and Fenton reactions leading to the generation of superoxide anion and hydroxyl radicals.[53] Ferrous ion chelating agents inhibit lipid peroxidation (by stabilization of transition metal), free radical generation, and resultant oxidative damage.[54] Both the *Arishta*/*Asava* and bacterial CFS exhibited good amount of

antioxidant activity in terms of scavenging the free radicals in the assay systems such as DPPH, hydroxyl, hydrogen peroxide, ABTS, nitric oxide and superoxide anion in addition to metal chelating and reducing power.

The profile of phytochemicals present in both *Balarishta* and *Chandanasava* such as phenolic compounds, tannins, flavonoids and phytosterol can harbor antioxidant compounds. GC-MS analysis also showed the presence of antioxidant compounds such as squalene, trans-squalene and lupeol (triterpenes), ß-sitosterol, (20R)-cholest-4-en-3-on, stigmasterol (phytosterols), 2-methoxy-4-vinylphenol (phenolics) and n-hexadecanoic acid (saturated fatty acid). ROS, reactive nitrogen species and reactive sulfur species have been linked to several diseases such as cancer, atherosclerosis, stroke, neurological disorders, renal disorders, rheumatoid arthritis, autoimmune diseases, inflammation and gastric ulcers.[51]

The antioxidant activity of *Balarishta* and *Chandanasava* was found to be less compared to the standards. It could be explained that standards are pure chemical forms, but the *Balarishta* and *Chandanasava* are chemically heterogeneous in nature. Moreover, the recommended dosage of consumption is around 15-30 ml twice per day. This may provide a considerable antioxidant support to the consumer of such *Arishta* and *Asava*.[23,24] Interestingly, the intrinsic microbes involved in the fermentation of *Balarishta* and *Chandanasava* also contribute for the antioxidant property of these medicines.[42] This will explain the traditional claim on the medicinal properties of *Balarishta* and *Chandanasava*. In addition to this, antioxidant compounds such as lupeol present in *Balarishta* also demonstrated to exhibit bioactivities such as anti-inflammatory (ameliorates rheumatism), anti-arthritic (ameliorates autoimmunity),[55] anti-ulcerogenic action (ameliorates gastric problems) and antinociceptive (ameliorates nervous disorders).[56] Presence of such compounds additionally reasons out the therapeutic property of such *Arishta.*

The better shelf-life-enhanced therapeutic properties, improvement in the extraction of drug molecules from the herbs and improvement in drug delivery of *Arishta* and *Asava* could be ascribed to the fermentation and biotransformation reactions mediated by the intrinsic microflora of these FTM. They also contribute to the antioxidant property of these medicines likely by the generation and sequestration of specific compounds. The plant materials act as a source of bioactive phytochemicals but microbes contribute for the increase in the bioactivity like antioxidant property.

Conclusion

The study indicated the presence of intrinsic microbes such as yeasts and bacilli that collectively function as a consortium. Dynamic changes in the profile of microbiota do occur during the course of this fermentation. These intrinsic microbes not only mediate fermentation but also perform biotransformation of phytochemicals. The profiles of phytochemicals are proven antioxidants. Moreover, the bacterial supernatants also exhibit antioxidant activity. Thus, the intrinsic microbes additionally contribute to the therapeutic efficacy of *Arishta* and *Asava*. Further studies are essential to demonstrate that the intrinsic microbes secrete specific compounds and contribute to the medicinal activities.

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Conflicts of interest

There are no conflicts of interest.

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