RASĀYAN *J. Chem.*
Vol. 16 | No. 2 |795-801| April - June | 2023
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

# FINDING OF POSITIVE ATTRIBUTING VALUES FROM A MEAT-DERIVED FOOD OF NORTHEAST INDIA, SATHU

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**ABSTRACT**

Fermented meat and meat-derived products are rich in significant microorganisms, along with their by-products, which provide quality, safety, and desired prophylaxis functions with them. This itself lends to a naturally good nutritional heritage and is considered an emerging biomarker product. Exploring their positive attributes may contribute prophylactic properties towards health care and profit to the food and feed industries. Our main objectives are to identify native potential bacteria in the regional indigenous meat-derived fermented food, Sathu. Besides, the study of their positive attributing properties of antibiotic sensitivity, anti-microbial, and cytotoxicity effects on the food using biological molecular methods is also necessary. Our findings from the 16S-rDNA sequencing method identified *Lactobacillus brevis* (KJR2) as the predominant microbial community followed by *Bacillus australimaris* (KJRc8). Phenotypic characterization revealed both the isolates possess the ability to exist in the harsh environment of gastric conditions. The two isolates were also susceptible to antibiotics, with more sensitivity towards ampicillin, penicillin, streptomycin, and neomycin. Confer of resistance to Penicillin was found to strain, KJR2. The two strains also revealed antagonistic effects against *B.subtilis*, *E.faecalis,* and *E. coli*. Moreover, the food showed cytotoxicity against cancer cell lines, when studied as a part of human efficacy.

**Keywords:** Anti-microbial, Antibiotic sensitivity, Cytotoxicity, Fermented food, Probiotic.
RASĀYAN*J. Chem.*, Vol. 16, No.2, 2023

## INTRODUCTION

Different varieties of meat-derived fermented products are created through fermentation by elaborating with meat and fat, mixed with meat and curing agents and spices. They are available and extended under different names like Salami, cured meat, sour meat, dry-cured hams, Jinhua and Xuanwei sausages in the Mediterranean region (Germany, United Nations, and Hungary) Salchichow, pepperoni in the USA, Chorizo in Europe.[1,2]Thus, the products of meat and meat represent representative groups of food on a national level.[3]In fact, the presence of lactic acid bacteria (LAB) in fermented meat products and because of their functional properties, fermented meats have received increased attention. The overall sequential actions during fermentation and their derivatives cause a series of changes in the structure, flavor, sensory characteristics, and other indicators in the products with nutritional benefits.[2] It has been confirmed that potential probiotic bacteria existing in traditional fermented meats have many organic purposes improving fitness stimulating profits, bio-preservation of delicate foods, bio-enrichment of nutritive values, defensive properties, and beneficial values.[4-6] The peptide, phytanic, conjugated linoleic acid; antioxidants, exopolysaccharides, lactic acid, organic acids, alcohols, and vitamins present in meat-derived proteins are responsible for prophylaxis properties. They have bioactivities other than essential amino acids and nutritional values.[7,2] In fact, fermented meat products have emerged as an appealing food biomarker for identifying health promoters. Among other microorganisms, the most common native microorganisms found in fermented meat include *Lactobacillus sakei, and Lactobacillus curvatus* followed by Coagulase-negative *Staphylococci* (CNS), *Kocuria* sppecis[8,9], *Micrococcus* spp. *Debaryomyces, Kodameae,* and *Wickerhrmomyces.*[10,11]Because of their probiotic properties, they were used as artificial inoculants to encourage the direct fermentation of meats. Similarly, in Northeast India diverse ethnic fermented meats are available to supplement their food necessity as well as for socio-cultural reasons. Northeast India lies in the eastern region of India constituted by seven states Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, and Tripura. About 8% of the total area of India is constituted by Northeast India which is about 3.1% of the Indian population.[12] Each indigenous group in the Northeast has its own specificities of fermenting meat and meat-derived products for


**http://doi.org/10.31788/RJC.2023.1628372**

preservation, flavor, and nutritional enhancement. Therefore, from the above mentions, we want to motivate positive values and exposure to indigenous fermented foods including involvement of name, inherent beneficial components, and health effects by consistent research. Only then it can break many stereotypical ideas, trigger the sense of values, and interests, and increase the eating of indigenous foods by modern consumers around the globe. However, it is yet unknown how much bacterial heterogeneity will be present in the accessible fermented meat products due to varied processing conditions, that are applicable throughout the Northeast.Thus, we are interested in isolating potential bacteria from fermented meat-derived products and testing their effectiveness on humans. We focus on Sathu, traditionally processed by kuki tribes of Northeast India to draw out its good qualities. Sathu is a fermented food made from pig fat. Our investigation will aid in obtaining the biological benefits and offer the product with credential values so that it may play a significant role in the food business similar to how commercial sausages do. Above all, the possibility of extinction by modernization's effect may lessen and enable showing how they are the potential to scale up the product while also inspiring the locals to start their own businesses.

## EXPERIMENTAL

### Preparation of Sample

Sathu is prepared in a conventional manner from pig fat. For the technique, a particular kind of fat known as caul fat is used. The animal carcass was properly prepared by skillfully removing the caul fat. Depending on taste, part of the subcutaneous fats can be accordingly for taste. In an anaerobic container or a conventional bamboo pot the extracted diced fats are cooked until they turn grey and then strung at a comfortable level above the home's customary hearth (4 feet high). The temperature of the jar is continuously maintained from the hearth by natural air and wood fire (35-45°C). After 7-20 days of incubation, the fermented pig fat is ready to use. Yet, superior flavor and consistency come from longer incubation. The entire fermentation process takes in Kuki village of Assam, India, and was packed and collected in the proper medium to ensure the freshness of the food sample.

### Isolation and Phenotypic Characterization of Isolates

About 100 mg of the food sample was transferred aseptically in a sterile container and homogenized into 10 ml of de Man-Rogosa Sharpe (MRS HiMedia) broth for 20 minutes using a vortex. The homogenized broth was anaerobically incubated in a BOD shaker (Impact Icon Instruments Company) at 37°C for 24h. The overnight samples were spread on MRS agar plates for 48h at 37°C after being diluted in a proper concentration. The plates were used to subculture and isolate bacterial colonies according to morphological appearance. The bacterial colonies were then reconstituted in MRS broth and cultured overnight. The broth cultures were again cultured on MRS agar plates containing 0.3% (W/V) CaCO3 after being serial. During incubation, a clear zone was seen around the bacterial colonies showing lactic acid reacts with $CaCO_3$.[13]From there possible LAB is chosen and tested for gram reaction, motility, catalase production, and cell morphology. After streaking on MRS agar, those heterogametic colonies were kept incubator for 48h at 37°C to produce pure colonies. The pure colonies were then kept at -20°C in 30% glycerol stock. Before any experimental tests, the stock cultures were sub cultured twice.

### Molecular Identification of Isolates

Genomic DNA for both bacteria was extracted with HiPurA TM Bacterial Genomic DNA Purification Kit (HiMedia) was used. Two universal primers combination of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT-3') are the primers used for amplification of the16S rDNA gene. The obtained sequences were compared using the Basic Local Alignment Tool (Blast:http: blast.ncbinlm.nih.gov/Blast.cgi) to see the homologous sequences.[14]

### Submission of New Sequences in NCBI GenBank

The 16S rDNA sequences retrieved from the genomic DNA of isolated bacteria KJRc8_C8 and KJR2_C9 were deposited in NCBI GenBank using Bank IT option. The sequence data used was ribosomal RNA.

### Determination of Optimum Temperature, pH, NaCl, and Bile Tolerance

The optimum temperature for both isolated bacteria was examined at various ranges of temperature (25°C, 30°C, 35°C, and 40°C). Freshly prepared overnight cultures were inoculated (100 µl in 9.9 ml of

MRS broth) and incubated at different temperatures. After 24h, cell mass was determined by measuring absorbance at O. $D_{600}$ using UV visible spectrophotometer (Thermo Scientific Multiskan spectrum). Further, the survival of isolates in an acidic medium was determined at different pHs ranging from 0 to 6, and 1N HCl was used to adjust the pH. Before and after 24h of incubation, the turbidity of the broth was measured at O. $D_{600.}$ The isolates were again tested for bile and NaCl tolerance by exposition in enriched MRS Broth containing bile salt (0.1-1%) and NaCl of varied concentrations (2-10%). Un-inoculated as well as enriched broth cells with bile and NaCl salt were measured for turbidity at O. $D_{600}$. The cell viability was then confirmed by spread plating on MRS agar plates. Each test was carried out in a triplicate pattern. The obtained data from temperature optimization, tolerance to pH, bile salts, and NaCl were analyzed from the data collected from three replicated tests.[14]

**Antibiotic Susceptibility Test of Isolates**

The disc diffusion method was performed using commercial antibiotic discs (HiMedia) on Muller Hinton agar (MHA) media(Hi-Media).[15] The sensitivity profiles were determined after 24h of incubation at 37°C by measuring the diameters of zone of inhibition (ZOI) in mm. The antibiotics used in the test were penicillin, neomycin, ampicillin, and streptomycin.

**Anti-Microbial Property Assessment**

The antimicrobial activity of KJR2 and KJRc8 against various food-borne pathogens was checked on MHA plates. On each spread plate of pathogens, 5 µl of solvent Dimethyl Sulfoxide (DMSO, HiMedia) along with 10 µl of different diluted concentrations (0 to 1000 µg/ml) of test cultures was diffused into the medium. One disc was loaded with only solvent as vehicle control and a ciprofloxacin disc (20µg/disc) was used as a positive control. After 24h incubation, the clear zone around the discs was measured.[16]

**Cytotoxicity Assay**

The cytotoxicity activity of the food sample was tested using (MTT) assay. The cancer cells lines of HCT116, A549, and HEPG2 $10^4$ cells were cultured in 96 well plates for 24h in DMEM (Dulbecco's Modified Eagle medium) with 10% FBS (Fetal Bovine Serium), 1% antibiotic solution and 5% $CO_2$ at 37°C with. The cells were then treated with extracted food samples ranging from 1-500 µg/ml. After 24h, MTT solution (250 µg/ml) was added to the cell culture and incubated for another 2h. The supernatant was discarded the and cell layer matrix was diluted in 100 µl Dimethyl Sulfoxide (DMSO) and measured using an Elisa reader (iMark, Biorad, USA) at 540 nm and 660 nm.[17]

## RESULTS AND DISCUSSION

**Morphological and Biochemical Characteristics**

Two representative bacteria were isolated from the food sample (Sathu) labeled as KJR2 and KJRc8. Both were phenotypically gram-positive and catalase negative. Morphologically KJR2 was rod-shaped while KJRc8 cocci shaped.

**The Bacterial Isolates Work Best at Gut Temperature and Exhibit Probiotic Properties**

The *in vitro* assessment of acidic, bile salts and NaCl tolerance revealed the survival rate of both strains. At pH 2, decreases in the number of cells were observed as compared with the incubation from pH 3 to pH 6 with maximum growth at pH 6. In our study, KJR2 and KJRc8 remained tolerant up to a concentration of 0.8% bile salt. Another important parameter of bacteria to scale probiotics is tolerance against toxic and osmotic shock in the human gut. In our case, both our strains showed significant growth at 2-4 % of NaCl concentration. They showed moderate sensitivity to increasing concentration of bile salts but unsafe for KJR2 at 1% bile salt as shown in Table-1. These show our isolates have the potential to sustain the human inhospitality stomach situation in order to colonize the intestinal system. The pH of the sample was moderately acidic (pH 4-4.5) despite the presence of a high population of LAB. This showed that the carbohydrates catabolism by *Bacillus* and *Lactobacillus species* had no effect on the pH food sample. This is an added advantage as the taste and delicacy of the food are compromised by the otherwise low pH.

**Molecular Identifications of the Isolates**

16S rDNA sequences showed KJR2 belonged to the genus *Lactobacillus* and species *brevis* with 99% similarity but not identical 100% to any of the reported strains and KJRc8 belongs to genus *Bacillus* and species *australimaris* with 97% similarity. *Lactobacillus brevis* KJR2 community was found dominated in the food sample. An accession number of MK729004.1 was given for KJR2_C9 and MK720731.1, KJRc8_C8 respectively. The phylogenetic tree analysis also showed the position of KJRc8_C8 and KJR2_C9 in different clades of their respective trees.

Table-1: Physico-Chemical Data of Isolated Strains KJR2 and KJRc8

| Biochemical test | Name of isolates | |
|---|---|---|
| | KJR2 | KJRc8 |
| Indole | - | + |
| Methyl red | + | + |
| Voges Proskauer's | - | - |
| Citrate utilization | + | + |
| Glucose | + | + |
| Arabinose | + | + |
| Sorbitol | + | + |
| Mannitol | + | + |
| Rhamnose | + | + |
| Sucrose | + | - |
| Temperature (°C ) optimum test | | |
| 25 | 0.088±0.03 | 0.316±0.01 |
| 30 | 0.410±0.00 | 0.408±0.04 |
| 35 | 0.720±0.01 | 0.756±0.05 |
| 40 | 0.163±0.06 | 0.291±0.05 |
| pH optimum test | | |
| 2 | 0.002±0.01 | 0.033±0.00 |
| 3 | 0.052±0.01 | 0.062±0.00 |
| 4 | 0.317±0.01 | 0.645±0.01 |
| 6 | 1.090±0.01 | 1.077±0.01 |
| 8 | 1.110±0.00 | 0.035±0.00 |
| 9 | 0.049±0.00 | 0.008±0.03 |
| Bile salt (%) | | |
| 0.1 | 0.516±0.06 | 0.651±0.01 |
| 0.3 | 0.475±0.12 | 0.610±0.00 |
| 0.6 | 0.386±0.34 | 0.521±0.03 |
| 0.8 | 0.330±0.04 | 0.471±0.12 |
| 1 | 0.013±0.00 | 0.446±0.65 |
| NaCl (%) | | |
| 2 | 0.519±0.01 | 1.251±0.02 |
| 4 | 0.203±0.01 | 0.749±0.02 |
| 6 | 0.025±0.00 | 0.339±0.00 |
| 8 | 0.013±0.00 | 0.293±0.00 |
| 10 | 0.007±0.00 | 0.027±0.00 |

**The Bacterial Isolates Exhibit High Susceptibility to Common Antibiotics**

The phenotypic profile of the antibiotic sensitivity test revealed that the isolates were both antibiotics susceptible. KJR2 showed the highest susceptibility to ampicillin while resistant to penicillin and intermediate to neomycin. In the case of KJRc8 high susceptibility was found in all the tested antibiotics compared to KJR2 with maximum sensitivity towards ampicillin followed by penicillin, streptomycin, and neomycin as in Table-2. This susceptibility to commonly used antibiotics such as ampicillin, streptomycin, and neomycin is a good indicator that Sathu can be consumed safely. This offers an opportunity for the KJR2 strain to use for patients receiving long-term antibiotic (penicillin) treatment.

Table-2:Antibiotic Susceptibility Test of Isolated Strains KJR2 and KJRc8

| Isolated species | Antibiotic | | Diameter of ZOI | Range | Interpretation |
|---|---|---|---|---|---|
| | Name | Amount on disc | | (R-I-S) | |
| | Penicillin | 10 units/disc | 30 mm | ≤14 15-16 ≥17 | S |
| KJRc8 (C8) | Streptomycin | 10 µg/disc | 25 mm | ≤11 12-14 ≥15 | S |
| | Ampicillin | 10 µg/disc | 34 mm | ≤11 12-13 ≥14 | S |
| | Neomycin | 30 µg/disc | 21 mm | ≤12 13-16 ≥ 17 | S |
| KJR2 (C9) | Penicillin | 10 units/disc | 13 mm | ≤14 15-16 ≥17 | **R** |
| | Streptomycin | 10 µg/disc | 17 mm | ≤11 12-14 ≥15 | S |
| | Ampicillin | 10 µg/disc | 21 mm | ≤11 12-13 ≥14 | S |
| | Neomycin | 30 µg/disc | 16 mm | ≤12 13-16 ≥ 17 | **I** |

**The Bacterial Isolates Have the Ability to Kill Food Borne Pathogens**

According to the antimicrobial assessment activity, the isolates have antimicrobial activities against common food-borne pathogens with the highest inhibition (12 mm) against *Bacillus subtilis* (*B.subtilis)* followed by *Enterococcus faecalis* (*E. Faecalis)* and *Escherichia coli* (*E.coli)* by both the isolates. Their zone of inhibition was found to be concentration dependent as in Fig.-1. The antimicrobial result revealed the tested isolates possibly produced bacteriocins, which serve as a barrier to inhibit the growth of harmful bacteria. This illustrates both KJR2 and KJRc8 could enhance the defense property that indirectly indicates the inherent properties of antimicrobial, and antifungal in the product.

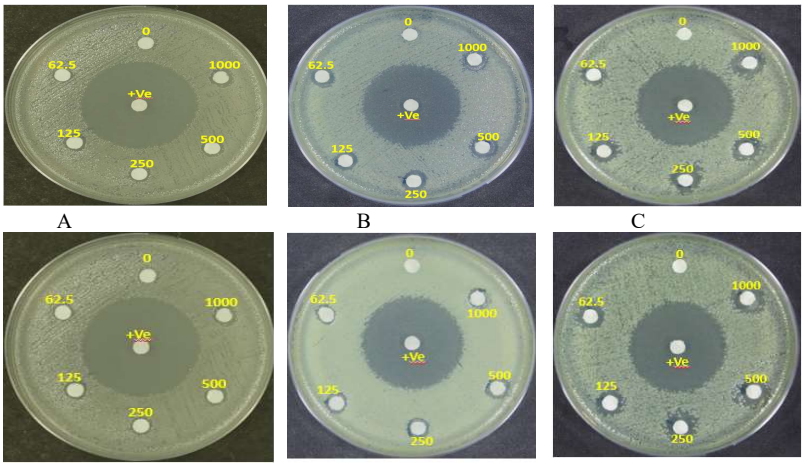


Fig.-1: Anti-microbial activities of KJRc8 and KJR2 respectively. A (*E. Coli),* B *(E. Faecalis),* and C *(B. Subtilis)*. The central clear zone represents positive control whereas the surrounding zones represent tests with different concentration of samples.

**The Fermented Food Does Not Induce Proliferation in Cancer Cells**

The cytotoxicity assay showed our food sample did not have an effect on the growth of cancer cell lines HCT116, A549 and HepG2. In all the tested cancer cell lines, there was the stability of cell number throughout. An increase in the sample concentration neither shows a decrease in the growth of cancer, Fig.-2. Our cytotoxicity study against metabolic cancer cell lines showed the food sample did not induced the formation of neither cancer cells nor help in proliferation.

## CONCLUSION

Our present study builds scientific evidence to consider Sathu as a functional product and specifically reveals its safety features. The product is mainly predominated by probiotic *Lactobacillus brevis* followed by *Bacillus australamaris*. Furthermore, the product has enumerated various health-promoting components during therapeutic analysis, which necessitated extensive research for all available nutrients in the product. As the consumption of probiotic bacteria in the form of sausages and other form of fermented foods has higher carrier rate of probiotics than those consumed in capsule form.[18] We therefore suggest as a good carrier of potential bacteria. Moreover, the strains KJR2 and KJRc8 can be subjected as

potential probiotic starter cultures or adjuncts in Hurdle technologies or the food sample can be supplemented for prevention of antibiotic potential.

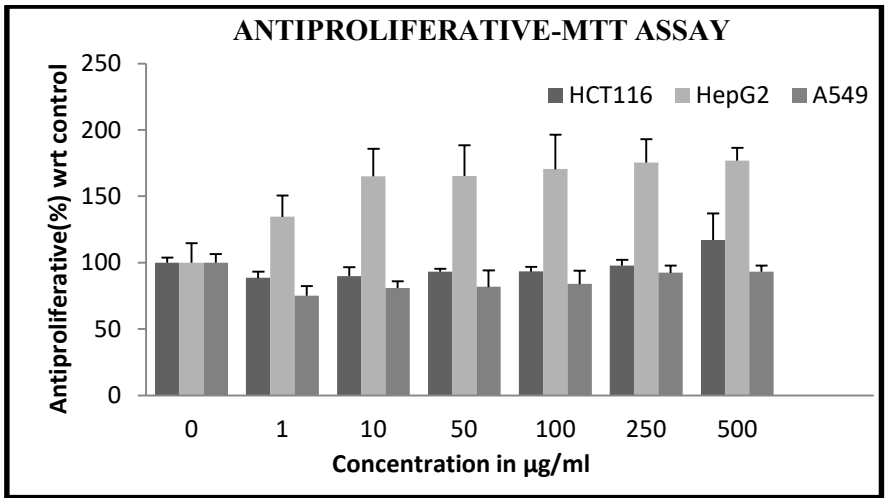


Fig.-2: Cytotoxicity Effects of Food Samples against Different Cancer Cell Lines

Further work identifying potential bioactive peptides would be of interest in connecting the consumption of food and control of metabolic diseases such as diabetes, hypertension, and other antioxidant properties. Current interest of creating principal group of fermented meat products, enabling the manufacture of bacteria with potential probiotic characteristics is fulfilled by breaking research barriers, accepting their attributing characteristics and most importantly compliance with the criterion of health safety are important factors to be taken into account.

## ACKNOWLEDGMENTS

The authors are grateful to the National Institute of Technology Arunachal Pradesh for extending assistance to the writers during our works.

## CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

## AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this work, R.Yumkhaibam was involved in mining data and writing and K. Lhouvum supports the editing/reviewing of the draft manuscript which was approved for publication. The ORCID ids of the writers listed below can be used to confirm their research profiles:

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