Isolation and characterization of *Lactococcus garvieae* from the fish gut for *in vitro* fermentation with carbohydrates from agro-industrial waste

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**A B S T R A C T**

This study focused on agro-industrial waste such as fruit peels by extracting prebiotics as a carbon source for lactic acid bacteria (LAB). Four strains of LAB were selected from *Oreochromis niloticus* (B2 and B3) and *Nemipterus japonicus* (R4 and R5), and identified as *Lactococcus garvieae* through 16S rRNA gene sequencing. The analysis of prebiotic characteristics revealed that all four strains were able to tolerate sodium chloride (up to 7%), bile salt (up to 3%), and broad range of pH (2–9). Further, analysis of polysaccharide contents in the agro-industrial waste materials such as peels of pineapple, orange, lemon, sugarcane, pomegranate, and sweet lemon revealed that the concentration ranged from 3.91–163.85 mg/g. It was observed that orange peels (20.38–140.99 mg/g), sweet lemon peels (22.03–161.93 mg/g), and pomegranate peels (38.19–163.85 mg/g) yielded maximum indigestible polysaccharide. Evaluation of symbiotic combination of prebiotic and probiotic revealed that *L. garvieae* strains had better fermentation efficiency with orange, sweet lemon, and pineapple compared to lemon, sugarcane, and pomegranate. In nutshell, different types of agro-industrial waste evaluated in this research were found to be a cheap and fermentable carbon sources for LAB. Further study should be conducted to analyze this symbiotic combination as feed supplements for fish in aquaculture as well as various fermentation industries. © 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Agro-industrial residues have drawn attention of scientists worldwide because of their potential as a raw material to prepare a range of industrial applications [1]. Agricultural systems reduce the environmental impact worldwide and ensure food security for future generations [2]. Agro-industrial wastes are solid organic residues produced during fruit harvest and food preparation for human and animal consumption. Agro-industrial waste includes nonedible parts of the fruits such as peel and seeds, which consist of enormous amount of nondigestible carbohydrates [3]. Wastes generated by fruit juice–processing industry are an excellent source of obtaining prebiotic oligosaccharides as novel food ingredients [4]. It is reported that citrus peel has more abundant functional ingredients such as fiber, oligosaccharides, and antioxidants [5]. Similarly, pineapple peel can be a source of dietary fiber as it contains plenty of cellulose, hemicellulose, and other carbohydrates [6]. Generally, nondigestible oligosaccharides used as prebiotic can be fermented by lactic acid bacteria (LAB) [7]. These compounds are directly associated with gastrointestinal microflora modification, which increases probiotic microbes and controls proliferation of pathogens [8]. LAB are a group of microbes used as probiotics, which exert significant health benefits on the host [9]. Information about prebiotic content in fruit peels and their *in vitro* fermentation with LAB is not available. A more detailed study is required to know the prebiotic potential and activity of agro-waste materials.

*Oreochromis niloticus* is the most farmed fish species because of its ability to feed on diverse organic materials, tolerance to a wide range of culture conditions, and wide availability to farmers. We speculated that probiotics from such fish might have diverse potential and might be able to ferment a range of organic compounds. On the flip side, no proper data on probiotics from *Nemipterus japonicus* are available. Considering this, it is important to characterize gut flora from *N. japonicus* and evaluate their fermentation capability with various agro-industrial wastes. LAB are normal residents of animal gut and can be used as probiotics and feed supplements. Apart from this, LAB have a wide range of applications in fermentation technology, food processing, as well as medicine, agriculture, bioremediation of environment, among others [10]. *Lactococcus garvieae* (L. garvieae) is from genus

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Lactococcus species previously known as the lactic acid producing members of streptococci [11]. There is no data available on symbiotic potential of L. garvieae with carbohydrates from organic waste. So, the present study was initiated with an objective to evaluate the prebiotic activity of six agro-industrial waste materials, that is, pineapple peel (Ananas comosus), orange peel (Citrus sinensis), lemon peel (Citrus lemon), sugarcane peel (Saccharum officinarum), pomegranate peel (Punica granatum), and sweet lemon peel (Citrus limetta), using LAB.

2. Materials and methods

2.1. Collection of fish and isolation of lactic acid bacteria from fish gut

O. niloticus (tilapia) and Nemipterus japonicus (red paree) were procured from Mindhora River, Bardoli. The fish samples were selected at random, collected in pre-sterilized polyethylene bags containing the habitat water, and transported to a laboratory. Fishes were washed with sterile distilled water to remove any undesired dust particles and dissected to open the gastrointestinal tract under laminar airflow conditions. The gastrointestinal tract was homogenized using sterile distilled water and centrifuged at 10,000 rpm for 10 min. After centrifugation, the supernatant was serially diluted and spread-plated on MRS agar plates (HiMedia). The plates were incubated at 37 °C for 24 h. The obtained isolates were further screened for their probiotic properties and four strains were selected for detailed characterization. The pure cultures were maintained on MRS agar slants at 4 °C for further study.

2.2. Assay for NaCl and bile tolerance

A basal MRS medium was used for the NaCl and bile salt tolerance analysis. The concentrations of NaCl and bile salt were 1 %–10 % and 1 %–3 %, respectively. An overnight culture of each Lactococcus strains was used as the inoculum. The suspension (0.1 mL) was inoculated into 9.9 ml MRS broth in each test tube. The tubes were incubated at 37 °C for 24 h. The turbidity of each tube was noted as an indication of growth or no growth.

2.3. Assay for pH tolerance

To evaluate the effect of pH on all four Lactococcus strains, 1% (v/v) overnight culture of was inoculated into the MRS broth with pH ranging from 2 to 9. The pH was adjusted with HCl and NaOH. The inoculated broths were incubated at 37 °C for 24 h. After 24 h of incubation, growth of the bacteria was observed for assessing the turbidity.

2.4. Assay for intrinsic antibiotic sensitivity

Colonies of all 4 Lactococcus strains were inoculated in MRS agar medium and the plate was allowed to dry before placing the diffusion discs containing antibiotics. The antibiotics used for the test were ampicillin (10 μg), gentamycin (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), cefalexin (30 μg), co-trimoxazole (25 μg), cefotaxime (30 μg), levofloxacin (5 μg), aztreonam (30 μg), imipenem (10 μg), amikacin (30 μg), and cefazidine (30 μg). The plates were incubated at 37 °C for 24 h and the zone of inhibition was measured and compared with the standard chart provided by the manufacturer.

2.5. Isolation of genomic DNA and 16 rRNA gene sequencing

The genomic DNA was extracted from the bacteria according to Sambrook [12]. Amplification of 16S rRNA gene was carried out by PCR (Eppendorf) using universal eubacterial primer set 63 F 5’-CAG GCC TAA CAC ATG CAA GTC-3’, 1387R 5’-GGG CGG AGT GTA CAA GGC-3’. Forward and reverse DNA sequencing reactions of PCR amplificon were carried out with 63 F and 1387R primers using BDT v3.1 Cycle Sequencing Kit on ABI 3730xl Genetic Analyzer. The homology of 16S rRNA gene sequence was aligned using BLAST program of the GenBank database (NCBI) and aligned to their nearest-neighbor sequences. A Phylogenetic tree was derived from sequences of 16S rRNA gene sequences using neighbor-joining method in MEGA X

2.6. Extraction of prebiotics from fruits peels

The six fruit peels were collected from a local juice center at Bardoli, Surat, Gujarat. The peel samples were chopped and finely ground in a blender. The grounded samples were then extracted with various solvents, as shown in Table 3. The ethanol extraction procedure was conducted at 30 °C for 3 days whereas water extraction was carried out at 30 and 90 °C for 3 h and 30 min, respectively. After the completion of the extraction procedure, samples were filtered through filter cloth. The filtrates were concentrated with the help of a rotary evaporator followed by freeze-drying. The samples were stored at 20 °C for further use.

2.7. Determination of prebiotic property of extracts

Sterile distilled water was used to prepare the dried-fruit peel extracts (10 % solutions w/v). Acidic digestion was carried out at 37 °C with HCl at pH 1 for 4 h and the reaction was terminated with NaOH [13]. Enzymatic digestion carried out using acid-digested solution at 37 °C with amylase in phosphate buffer solution (20 mM) at pH 6.9 for 6 h and the reaction was terminated by heating at 80 °C for 10 min [14]. Amounts of indigestible polysaccharides in the extracts were determined by analyzing reducing sugar contents (mg/g) using the modified dinitrosalicylic acid method [15]. This was followed by analysis of total sugar contents (mg/g) with the modified phenol sulfuric method [16]. The following formula was used to calculate the indigestible polysaccharide content (mg/g dry extract) in the extracts:

\[ \text{Indigestible polysaccharides (mg/g) = Total sugar after acid-enzyme digestions (mg/g) - Reducing sugar before the digestions (mg/g)} \]

On the basis of the extract yields and indigestible polysaccharides in the extracts, all samples were chosen for further studies. Further, the amount of carbohydrates was also evaluated after H2SO4/phenol digestion following Masuko et al. [17].

2.8. Evaluation of indigestible carbohydrates (prebiotics) for use as C source by Lactococcus garvieae (probiotics)

The fermentation of prebiotic and its utilization as C source by probiotics were evaluated in MRS broth. The MRS broth was reconstituted with indigestible carbohydrates extracted from fruit peels in place of glucose (1 % w/v). Commercial prebiotics Inulin and Fructo-oligo saccharide (FOS) (1 % w/v) were used as positive control. Furthermore, evaluation was also carried out for Escherichia coli, Staphylococcus aureus, and Bacillus subtilis to check whether prebiotics were supporting the growth of pathogens. The MRS broth tubes were inoculated with overnight-grown cultures from each strain at a final concentration of 10 μL/mL and incubated at 37 °C for 24 h. The turbidity of the culture was measured by taking absorbance at 600 nm in a spectrophotometer.

3. Results and discussion

3.1. Lactic acid bacteria from fish gut

In this study, efforts were made to isolate potential bacterial strain that can serve as a probiotic as well to manage the agro-
industrial waste by fermentation. The gastrointestinal microbiota plays an important role in host health and metabolism. Therefore, a study was conducted to isolate LAB from O. niloticus and N. japonicus fish gut. Four potential strains were selected from O. niloticus (B2 and B3) and N. japonicus (R4 and R5) based on the probiotic characteristics and identified through 16S rRNA gene sequencing as L. garvieae. BLAST analysis confirmed that the homology of the isolates were identical to that of L. garvieae. The 16S rRNA gene sequences identified in this study were deposited in the GenBank database (www.ncbi.nlm.nih.gov/GenBank/index.html) and accession numbers were assigned (Table 1). Most gut microbiota may not be cultivable under in vitro conditions used in this study; there will be a considerable difference to the actual microbiome. In a similar research, AbdelFatah and Mabhou reported L. garvieae of dairy origin as a probiotic for controlling the pathogenic S. aureus in O. niloticus [18]. L. garvieae is also reported to produce bacteriocin [19,20].

Tolerance to low pH and bile salts during transit is essential for LAB to survive through the gastrointestinal tract and exert their beneficial effects [21]. The isolated L. garvieae strains from the fish gut were able to tolerate 1 ‰–3 % bile as well as pH range between 2 and 9 (Table 2). The motives for choosing this pH range were to determine whether L. garvieae strains can grow in acidic and alkaline environments and to determine the optimum pH for luxuriant growth. From the experimental results, it was found that the isolated L. garvieae strains from fish gut are able to survive in extreme acidic pH (pH 2–3) and basic pH (pH 7–9). Maximum growth of isolated Lactococcus from both fish was observed at pH 7–9 (Table 2). Our results are in accordance with those reported by Zhang et al., who found an acid pH-tolerant strain of L. garvieae B301, which was capable of surviving in the gastrointestinal tract [22]. Zhang et al. reported that the L. garvieae strain B301 could improve the growth performance and health in broiler chickens [22]. NaCl is an inhibitory substance that may inhibit the growth of certain types of bacteria. In this study, it was noted that all L. garvieae strains were able to grow at higher salt concentration (up to 7 %). These properties indicated that all L. garvieae strains are ideal probiotic candidates. Our results are similar to those reported by Xu, Luo, Bao, Liao, and Wu (2018), that L. garvieae subspecies strain have probiotic properties [23].

Commercially, LAB are used extensively and specifically selected to prevent antibiotic resistance and exchange of transferable resistance genes [24]. The antibiotic susceptibility test showed that L. garvieae was sensitive to ampicillin, tetracycline, ciprofloxacin, cefalexin, co-trimoxazole, cefotaxime, aztreonam, ceftazidine, and imipenem but resistant to gentamycin, levofloxacin, and amikacin (Table 3). A similar research reported resistance of L. garvieae against oxytetracycline, erythromycin, amoxicillin, and florfenicol [25]. Differences in antibiotic resistance profiles among various L. garvieae strains help in understanding the basis of optimally selecting probiotics for selective fermentation.

### Table 1
Identification Lactococcus based on 16S rRNA gene sequencing.

| Isolate Code | Identified As       | % Homology | Source          | Accession Number |
|--------------|---------------------|------------|-----------------|------------------|
| B2           | Lactococcus garvieae| 99 %       | Oreochromis niloticus | MF351800        |
| B3           | Lactococcus garvieae| 99 %       | Oreochromis niloticus | MF351801        |
| R4           | Lactococcus garvieae| 99 %       | Nemipterus japonicus | MF351802        |
| R5           | Lactococcus garvieae| 99 %       | Nemipterus japonicus | MF351803        |

### Table 2
Evaluation of probiotic properties of Lactococcus garvieae.

| L. garvieae | Bile salt | NaCl | pH 1 | pH 2 | pH 3 | pH 4 | pH 5 | pH 6 | pH 7 | pH 8 | pH 9 |
|-------------|-----------|------|------|------|------|------|------|------|------|------|------|
| B2          | ++        | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| B3          | +++       | -    | +++  | +++  | +++  | +++  | +++  | +++  | +++  | +++  | +++  |
| R4          | +++       | -    | +++  | +++  | +++  | +++  | +++  | +++  | +++  | +++  | +++  |
| R5          | +++       | -    | +++  | +++  | +++  | +++  | +++  | +++  | +++  | +++  | +++  |

+++ Good Growth; ++ Moderate Growth; + Poor Growth; - No Growth.

### Table 3
Susceptibility profiles of Lactococcus garvieae against antibiotics.

| Antibiotic         | Concentration (μg) | Zone of Inhibition (mm) |
|--------------------|--------------------|-------------------------|
|                    | B2  | B3  | R4  | R5  |
| Ampicillin (AMP)    | 10  | 16  | 25  | 23  | 13  |
| Gentamicin (GEN)    | 10  | 13  | 14  | R   | 12  |
| Tetracycline (TE)   | 30  | 21  | 30  | 26  | 20  |
| Ciprofloxacin (CIP) | 05  | 19  | 23  | 15  | 23  |
| Cefalexin (CN)      | 30  | 25  | 34  | 11  | 24  |
| Co-Trimoxazole (COT)| 25  | 19  | 27  | 14  | 14  |
| Cefotaxime (CTX)    | 30  | 15  | 28  | 25  | 18  |
| Levofloxacin (LE)   | 05  | 15  | 18  | 25  | R   |
| Aztreonam (AT)      | 30  | 20  | 29  | 28  | 15  |
| Imipenem (IPM)      | 10  | 14  | 30  | 18  | 13  |
| Amikacin (AK)       | 30  | 13  | 28  | 23  | R   |
| Ceftazidine (CAZ)   | 30  | 18  | 27  | 20  | 19  |

R- Resistant.
analyzed in the present study had a high potential for further expansion as commercial prebiotics. Sweet lemon peel recorded to have highest 136.11 mg/g of total indigestible carbohydrates with water at 90 °C for 30 min and lowest 46.18 mg/g with 95 % alcohol (Table 4). The amount of indigestible carbohydrates extracted from pineapple peel with water (90 °C at 30 min) was 118.57 mg/g whereas that from orange peel was 117.33 mg/g. Sugarcane peel is the waste from sugar industries but it is rich in carbohydrates. Indigestible carbohydrates extracted from sugarcane with water (90 °C at 30 min) were 73.86 mg/g. Most of the peels we used in this study had shown good amount of prebiotics that can be selectively used for fermentation with LAB. Overall data suggest that extraction in boiling water could be a potential method followed by alcohol for prebiotic extraction.

3.4. Indigestible carbohydrates (prebiotics) as carbon source by L. garvieae (prebiotics) Garvieae (prebiotics)

Several studies have shown that LAB strains are substrate specific and ferment prebiotic carbohydrates in a selective manner [30,31]. Further, scant information is available about which ingestible carbohydrate is the most suitable substrate for selective growth of specific strains. Many quantitative methods have been developed to determine the functional activity of prebiotics during fermentation [32–34]. Therefore, in the present study we

| Plant          | Extraction | Temperature | Time  | Carbohydrate concentrations (mg g⁻¹) | Acid/ enzyme digestion | H₂SO₄/Phenol digestion (mg/g) | Indigestible Carbohydrates |
|----------------|------------|-------------|-------|-------------------------------------|------------------------|-----------------------------|-----------------------------|
| Sweet Lemon    | Water      | 30 °C       | 3 h   | 25.82 ± 1.91                        | 161.93 ± 8.01          | 112.22 ± 6.06               |
|                | Water      | 90 °C       | 30 min| 27.34 ± 2.01                        | 139.56 ± 6.93          | 136.11 ± 7.83               |
|                | 95 % Alcohol | 30 °C       | 3 days| 22.03 ± 1.67                        | 64.39 ± 4.52           | 42.36 ± 2.91                |
| Sugarcane      | Water      | 30 °C       | 3 h   | 23.44 ± 1.78                        | 61.0 ± 3.98            | 37.67 ± 2.95                |
|                | Water      | 90 °C       | 30 min| 19.10 ± 1.23                        | 92.96 ± 4.78           | 73.86 ± 3.74                |
|                | 95 % Alcohol | 30 °C       | 3 days| 50.35 ± 3.58                        | 75.05 ± 4.05           | 24.70 ± 1.97                |
| Pineapple      | Water      | 30 °C       | 3 h   | 14.97 ± 1.03                        | 96.68 ± 4.95           | 81.70 ± 4.63                |
|                | Water      | 90 °C       | 30 min| 21.92 ± 1.54                        | 140.49 ± 6.83          | 118.57 ± 6.82               |
|                | 95 % Alcohol | 30 °C       | 3 days| 29.73 ± 2.76                        | 122.89 ± 6.03          | 93.16 ± 4.25                |
| Orange         | Water      | 30 °C       | 3 h   | 25.93 ± 2.17                        | 67.24 ± 3.74           | 41.31 ± 2.71                |
|                | Water      | 90 °C       | 30 min| 23.65 ± 1.74                        | 140.99 ± 7.47          | 117.33 ± 6.89               |
|                | 95 % Alcohol | 30 °C       | 3 days| 34.94 ± 2.91                        | 35.32 ± 2.86           | 20.38 ± 1.92                |
| Lemon          | Water      | 30 °C       | 3 h   | 3.91 ± 0.58                         | 50.01 ± 3.07           | 19.60 ± 2.02                |
|                | Water      | 90 °C       | 30 min| 17.58 ± 1.05                        | 37.18 ± 2.97           | 46.10 ± 3.09                |
|                | 95 % Alcohol | 30 °C       | 3 days| 14.00 ± 0.98                        | 60.17 ± 3.75           | 46.18 ± 2.96                |
| Pomegranate    | Water      | 30 °C       | 3 h   | 38.19 ± 2.78                        | 62.65 ± 4.02           | 24.46 ± 2.02                |
|                | Water      | 90 °C       | 30 min| 72.05 ± 4.53                        | 99.26 ± 5.17           | 27.91 ± 2.98                |
|                | 95 % Alcohol | 30 °C       | 3 days| 30.27 ± 1.69                        | 163.85 ± 8.08          | 133.58 ± 7.18               |
compared the growth of L. garvieae on various peel extract prebiotics along with pathogens such as E. coli, S. aureus, and B. subtilis (Table 5).

The highest probiotic growth was observed with carbohydrates extracted from orange, sweet lemon, and pineapple for all the L. garvieae strains tested in this study. On the flip side, it was noted that these three prebiotics were supported very less growth of pathogens such as E. coli, S. aureus, and B. subtilis. This is an important attribute of extracted carbohydrates as it indicates that it will only support the probiotic multiplication instead of pathogens inside GIT of fish. Utilization of particular carbohydrate by bacteria requires the presence of specific hydrolysis and transport systems [31,35]. This indicates that orange, sweet lemon, and pineapple are a promising source of prebiotics and can be tested further for their symbiotic application in aquacultures as well as various fermentation industries. Symbiotics exert a beneficial effect in gastrointestinal tract by increasing the count of LAB and restricting the growth of potential pathogens [36]. For positive control, we used commercial prebiotics (Inulin and FOS) and surprisingly we found that the growth of L. garvieae strains was comparable with peel-extracted prebiotics. In the case of glucose, equal growth was noted for probiotics and pathogens may be because of the metabolic diversity of L. garvieae. Overall, it is noted that there was a considerable variation in growth for different prebiotics used by a single strain.

4. Conclusion

In this study, four strains of LAB L. garvieae were successfully characterized as prebiotics from O. niloticus and N. japonicus fish gut. Further, we reported that different agro-industrial waste materials can be used as a source of carbohydrate for various fermentation processes. Fruit peels contain considerable amounts of polysaccharides that are prebiotics candidates as they can withstand the acidic conditions and enzymatic treatment. Among the six peel sources, orange, sweet lemon, and pineapple were found to have the highest potential for further research and development. The prebiotic properties of all of them were confirmed by selective in vitro symbiotic fermentation with probiotics strain L. garvieae. Further study should be conducted under in-vivo conditions for its possible application in aquaculture.

Author contributions

Conceived and designed the experiments: PP, RK, NA. Performed the experiments: BP, RS, BJ. Analyzed the data: PP, NA. Wrote the paper: PP, RK, NA.

Ethical statement

This is to certify that the submitted manuscript is original, unpublished and not under simultaneous consideration by another journal.
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