General methods to isolate, characterize, select, and identify fructophilic lactic acid bacteria from fructose-rich environments – A mini-review

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Abstract. Fructophilic lactic acid bacteria are a group of newly discovered lactic acid bacteria. Despite their potential application as probiotics in the food industry, exploration of ecological niches to discover new fructophilic lactic acid bacteria is scarce, and information that concisely describes the practical aspects of their discovery process is limited. In this mini-review, we focus on methods that have been developed to discover fructophilic lactic acid bacteria from fructose-rich environments such as flowers and bee products. First, we briefly introduce the definition, classification, diversity, and ecological niches of fructophilic lactic acid bacteria. Next, we discuss the unique characteristics that distinguish fructophilic lactic acid bacteria from other microorganisms. Finally, we outline the principles and steps to isolate, characterize, select, and identify fructophilic lactic acid bacteria. The discovery of fructophilic lactic acid bacteria with unique characteristics could provide an impetus for the development of probiotics from fructophilic lactic acid bacteria.

1. Introduction
Lactic acid bacteria are a diverse group of bacteria that produce lactic acid as the main product of sugar fermentation. Historically, lactic acid bacteria have been closely associated with fermented foods and the beneficial health effects they provide, although some members, such as streptococci and enterococci, are known to be pathogenic [1]. Taxonomically, lactic acid bacteria may comprise around 20 genera (all Gram-positive), including the commonly-known genus Lactobacillus that forms one of the core genera of the group [1]. In addition to their rich diversity, lactic acid bacteria are widely distributed. They have been found across distinct ecological niches, from plants to animals, interacting and co-evolving with their hosts, inhabiting them transiently or permanently while exerting dedicated or multiple functions [2]. Different ecological niches will have different conditions, including the type and amount of carbon sources available. These specific conditions will shape the type of lactic acid bacteria that inhabit certain niches, e.g., in fructose-rich environments, a specific group of lactic acid bacteria called fructophilic lactic acid bacteria might commonly be found.

Fructophilic lactic acid bacteria have been isolated from flowers, fruits, fermented fruit products, the gastrointestinal tracts of insects with fructose-based diets such as bees, and bee products [3,4]. Interest in fructophilic lactic acid bacteria has grown and stems from their unusual metabolism and probiotic potential [5,6]. With their unique properties, fructophilic lactic acid bacteria may find useful applications...
for paratransgenesis to control diseases in honey bees and for functional foods [7]. Given their vital ecological role and potential applications, it is important to understand the diversity of fructophilic lactic acid bacteria from different niches. To capture a complete fructophilic lactic acid bacteria consortium, the isolation of fructophilic lactic acid bacteria from different niches would require different isolation methods because, unless the conditions of their natural environment are met, there will be less than 1% of the microbes that can be cultured (referred to as the great plate count anomaly). Although culturing fructophilic lactic acid bacteria under the natural conditions of their niche is not possible, methods have been developed and used to isolate “culturable” fructophilic lactic acid bacteria under general laboratory conditions. Because the field of study of fructophilic lactic acid bacteria is relatively new, information regarding methods to discover fructophilic lactic acid bacteria from fructose-rich environments is still scattered. To help inform researchers new to the field, here we review the growth and morphological characteristics of fructophilic lactic acid bacteria, the media composition used for their isolation, and the relevant methods used to isolate, characterize, select, and identify them. Before going into the general principles and steps to discover fructophilic lactic acid bacteria, we will first discuss the characteristics that distinguish fructophilic lactic acid bacteria from other microorganisms. This knowledge is essential for isolating fructophilic lactic acid bacteria.

2. Unique characteristics of fructophilic lactic acid bacteria

One of the main distinguishable characteristics of fructophilic lactic acid bacteria is that they grow well on fructose but not on glucose, unlike most other lactic acid bacteria [5]. Based on their growth characteristics, fructophilic lactic acid bacteria can be divided into two groups: obligate and facultative. Obligate fructophilic lactic acid bacteria require the presence of external electron acceptors such as pyruvate or oxygen for growth on glucose, whereas facultative fructophilic lactic acid bacteria can grow on glucose without the electron acceptors but at a delayed rate [5]. Members of obligate fructophilic lactic acid bacteria include all Fructobacillus species, Lactobacillus kunkeei, and L. apinorum [7] whereas members of facultative fructophilic lactic acid bacteria include L. florum and L. brevis [8,9].

Another characteristic of fructophilic lactic acid bacteria is that they can grow well under both aerobic and anaerobic conditions when supplemented with fructose but only under aerobic conditions when supplemented with glucose—they either do not grow or produce pinpoint colonies (0.1 mm in diameter) on glucose under anaerobic condition after 2–5 days [4,10]. Besides fructose and glucose, fructophilic lactic acid bacteria can only metabolize a limited number of carbohydrates, which include sucrose and trehalose [11]. Based on their metabolism, fructophilic lactic acid bacteria are classified as heterofermentative LAB, producing lactic acid, acetic acid, and a trace amount of ethanol due to the lack of adhE gene that encodes for alcohol/acetaldehyde dehydrogenase [12–14].

As mentioned above, fructophilic lactic acid bacteria inhabit fructose-rich niches and have adapted to grow under the environmental conditions of and dynamic interspecies interaction within their niche. For isolation purposes, information regarding the optimum growth conditions for culturing fructophilic lactic acid bacteria is useful but not mandatory. Growth optimization studies for fructophilic lactic acid bacteria have scarcely been performed. One study reported that Lactobacillus strains from wild bees and flowers have optimum growth temperature at 30–35°C and pH 5–6 when cultured in MRS media supplemented with 20% D-fructose under static condition [15]. To isolate fructophilic lactic acid bacteria from their natural environment, one can use a general growth condition described in the literature for setting up the media composition, temperature, pH, aeration, and agitation. One can also expect similar growth characteristics of fructophilic lactic acid bacteria under the specific growth conditions described. For example, Lactobacillus and Fructobacillus strains isolated from fresh flowers and fruit peels can grow exponentially in one day and reach a stationary phase after two days when cultured in GYP media at 30°C under 120 rpm agitation [5]. Fructobacillus strains isolated from natural resources can grow exponentially in 4 hours and reach the stationary phase after 12 hours when cultured in MRS media supplemented with 1% D-fructose at 30°C under static conditions [16]. In general, the growth characteristics of fructophilic lactic acid bacteria described in the literature can be used as a reference for setting up the condition for fructophilic lactic acid bacteria isolation. As a guideline,
fructophilic lactic acid bacteria can be cultured in fructose-supplemented media at moderate temperature, slightly acidic pH, and under aerobic or anaerobic conditions. A shortlist of fructophilic lactic acid bacteria growth characteristics are given in table 1.

Table 1. Growth characteristics of notable fructophilic lactic acid bacteria isolated from different niches.

| Species         | Source                        | Growth Temperature (°C) | pH          | Salt (%) | Fructose (%) | Ref.     |
|-----------------|-------------------------------|-------------------------|-------------|----------|--------------|----------|
| L. apinorum     | Honey bees and their products | 15–50                   | 3.0–12.0    | NA       | NA           | [14,17]  |
| L. brevis       | Flowers                       | 30                      | 6.8         | NA       | NA           | [9]      |
| L. forum        | Flowers and fruits            | 30                      | 4.0–8.0     | 5        | 30           | [8,18]   |
| L. Kunkle       | Wine, honey bees, and flowers | 25–30                   | 3.7–8.0     | 5        | 30           | [5,11,19–23] |
| F. durionis     | Fermented foods               | 5–35 (optimum at 30)    | Optimum at 6.5 | 8        | 40           | [24–26] |
| F. ficulneus    | Fruits                        | Optimum at 30           | Optimum at 6.5–7.0 | 5        | 40           | [26]     |
| F. fructosus    | Wine, honey bees, and flowers | 6–40 (optimum at 30)    | Optimum at 6.5–7.0 | 5        | 40           | [23,25,27,28] |
| F. pseudoficulneus | Fermented fruits              | Optimum at 30           | Optimum at 4.8–8.5 | 5        | 40           | [23,25] |
| F. tropaeoli    | Flowers and fruits            | Optimum at 30           | 4.0–8.0     | 2.5      | 30           | [10,26]  |

*Positive but delayed fermentation
NA, information not available

3. Fructophilic lactic acid bacteria screening principles and methods

In general, the screening of fructophilic lactic acid bacteria involves four main steps: isolation, characterization, selection, and identification. Note that these four steps are not necessarily sequential (the steps are interrelated to and overlap with each other) but for simplicity they will be discussed in sequential subsections following common practice. The aim of isolation is to separate different fructophilic lactic acid bacteria species from one another. After isolation, each fructophilic lactic acid bacteria species can be characterized to determine its morphological properties, growth characteristics, biochemical properties, and probiotic potential. These data can then be compared and used to select fructophilic lactic acid bacteria isolates based on the most desirable properties. Selected fructophilic lactic acid bacteria isolates can then be sequenced to identify their phylogenetic positions. In the following subsections, we outline the principles and methods to isolate, characterize, select, and identify fructophilic lactic acid bacteria species from fructose-rich environments [3–5,21,23,29,30].

3.1. Isolation

The isolation of fructophilic lactic acid bacteria species from the environment starts with collecting fructose-rich samples. The sampling should be performed aseptically and different sets of sampling apparatus should be used for collecting different samples to avoid cross-contamination. The latitude and longitude of the sampling location may be recorded for reference. Photographs of the samples may be documented for reporting purposes. The duration between the time of sampling and culturing should be set to a minimum to avoid sample deterioration, which could affect the composition of the fructophilic lactic acid bacteria consortium. Thus, media preparation for culturing fructophilic lactic acid bacteria should be done before sampling. An example of media composition that can be used to screen fructophilic lactic acid bacteria from fructose-rich environments is given in Table 2.
Table 2. Fructose/glucose yeast peptone (F/GYP) media composition for fructophilic lactic acid bacteria isolation [5].

| Component                  | Concentration (g/L) | Function                                                                 | Ref.        |
|---------------------------|---------------------|--------------------------------------------------------------------------|-------------|
| 1. F/GYP broth media      |                     |                                                                         |             |
| D-fructose/D-glucose      | 10                  | D-fructose is the substrate preference of fructophilic lactic acid bacteria. D-glucose is used for growth characterization. | [5,25]      |
| Yeast extract             | 10                  | Yeast extract and peptone are used to improve growth because they contain essential nutrients | [5,31]      |
| Peptone                   | 5                   |                                                                         |             |
| CH₃COONa.3H₂O             | 2                   | Sodium acetate is used as a buffer and to suppress the growth of spoilage bacteria |             |
| Tween 80                  | 0.5                 | Tween 80 contains primary fatty acid to enhance growth and protect against environmental stressors |             |
| MgSO₄.7H₂O                | 0.2                 | Minerals serve as micronutrients for promoting growth                    |             |
| MnSO₄.4H₂O                | 0.01                |                                                                         |             |
| FeSO₄.7H₂O                | 0.01                |                                                                         |             |
| NaCl                      | 0.01                | Sodium chloride is used to select salt-tolerant bacteria                  |             |
| NaN₃                     | 0.05                | Sodium azide is used to inhibit Gram-negative bacteria                    |             |
| Cycloheximide             | 0.05                | Cycloheximide is used to inhibit the growth of fungi                      | [32]        |
| 2. F/GYP agar media       |                     |                                                                         |             |
| Agar                      | 12                  | Agar is added to the media to form a solid structure                     | [5]         |
| CaCO₃                     | 5                   | Calcium carbonate will be hydrolyzed by lactic acid and form a clear zone as an indicator of lactic acid bacteria |             |

Once collected, environmental samples can be aseptically crushed (using sterile micro-tube sample pestles), added with FYP media (Table 2; the volume used may vary depending on the sample size but 1 mL will typically suffice), and incubated at a desirable temperature (Table 1; typically, at 30°C but room temperature will also work) under aerobic (with gentle agitation at around 100 rpm) or anaerobic condition (static) for 24 hours. This step will enrich Gram-positive fructophilic bacteria present in the environmental samples. After incubation, 100–500 μL of the enriched culture can be inoculated into 20–100 mL of selection media (FYP with 30–40% D-fructose; Table 1) to enrich fructophilic lactic acid bacteria, which are known to be osmotolerant [25], and incubated as above. After the second enrichment, 100 μL of the culture can be serial-diluted, spread onto FYP agar media (Table 2), and grown for 2–5 days. Fructophilic lactic acid bacteria colonies will typically grow to around 1–2 mm in diameter with smooth edge and beige color (1). A clear zone can be observed around the colonies as a result of calcium carbonate hydrolysis by lactic acid. The shape and size of the colonies as well as the size of the clear zone can be used as a basis for selecting colonies to be further characterized.

Figure 1. Examples of fructophilic lactic acid bacteria colonies isolated from bee bread grown on FYB agar. Note that morphologically, fructophilic lactic acid bacteria colonies (1–4) may appear similar but biochemically, each isolate display different properties and thus should be independently sequenced for identification. The morphological characteristics of fructophilic lactic acid bacteria is given in Table 3.
3.2. Characterization
To obtain fructophilic lactic acid bacteria with desirable properties, isolated colonies can be picked and characterized. The first characterization would be testing the ability of the colonies to grow in FYP but not in GYP at 30°C or room temperature under anaerobic condition for 24 hours. One can use 5 mL of media per colony (the amount taken should be consistent between colonies) and compare the absorbance of the culture at 600 nm or quantitatively count the colony forming unit per mL of culture (cfu/mL) on plate. This step will determine whether the colonies picked are fructophilic, preferring fructose over glucose, or not. After selection, a more detailed characterization of selected fructophilic lactic acid bacteria candidates can be performed. Note that the technical details regarding the assay may vary and is beyond the scope of this article. For details, readers are referred to respective lab protocols.

3.2.1. Morphological properties. As noted earlier, all fructophilic lactic acid bacteria species are Gram-positive, and the isolation process also favours the selection of Gram-positive bacteria. Thus, the first characteristic of the fructophilic lactic acid bacteria candidates to be confirmed is their type of cell wall using Gram staining. The ability to form spores can be tested using endospore staining. Afterwards, the shape and dimension of the candidates may be examined using electron microscopy (EM). Because morphological data can be used to help identify the bacteria, this step may be considered as part of the identification process. A short list of morphological characteristics of fructophilic lactic acid bacteria is given in Table 3.

Table 3. Morphological characteristics of notable fructophilic lactic acid bacteria isolates.

| Species          | Colony Description                  | Colony Size (mm) | Cell Size (µm) | Cell Occurrence                  | Ref. |
|------------------|-------------------------------------|------------------|----------------|----------------------------------|------|
| L. apinorum      | Smooth to rough surface, circular and raised | 3–4              | 0.5–0.8 × 1.5–6.0 | Non-motile rods, single or in pairs | [17] |
| L. brevis        | Beige, circular with smooth surface  | 0.5–2            | 0.7–1.0 × 2–4 | Non-motile rods with rounded ends, single and in short chains | [29] |
| L. florum        | Beige, smooth surface               | 1–2 (anaerobic)  | 0.8 × 1.5–7   | Non-motile rods, single or in pairs and chains |         |
| L. kunkeei       | Opaque, concave                     | 1–2              | 0.5 × 1–1.5   | Single, pairs or chains          | [18] |
| F. durionis      |                                    |                  | 0.5 × 2–6     |                                  |      |
| F. ficulneus     |                                    |                  | 0.5 × 1.5–3   | Non-motile rods, single or in pairs | [30] |
| F. fructosus     | White, smooth surface               | 1–2 (aerobic); 0.1–0.2 (anaerobic) | 0.5 × 0.8 × 2–8 | Non-motile rods, single, in pairs or in chains | |
| F. pseudoficulneus |                                  |                  | 0.5 × 1–5     |                                  |      |
| F. tropaeoli     |                                    |                  | 0.8 × 1.5–6   |                                  |      |

3.2.2. Biochemical properties. Biochemical characterization of fructophilic lactic acid bacteria species typically includes: (1) carbohydrate fermentation using analytical profile index (API) strips; (2) enzymatic activities using API strips; (3) gas production using inverted Durham tubes; (4) fermentation end-products (ethanol, lactic acid, and acetic acid) using respective assay kits; and (5) antibiotic resistance using standardized disk diffusion assay [33]. In addition, quantitative analyses using high performance liquid chromatography (HPLC) and mass spectrometry (MS) may also be performed to provide more detailed results. Similar to morphological characterization, biochemical analyses may also be considered as part of the identification process because the data provides additional description of the bacteria.

3.2.3. Probiotic potential. The potential application of fructophilic lactic acid bacteria as probiotics has attracted interest in evaluating their beneficial properties. The evaluation of probiotics for use in food has been outlined by FAO/WHO and includes in vitro tests for functional characterization and safety
assessment [34]. In order to exert their function, probiotics must be able to colonize the gastrointestinal tract of their host. Thus, it is mandatory that probiotics pass the following criteria: survive during gastric transit, tolerate bile salts, and adhere to gut epithelial tissue [35]; before characterizing their functional properties and assessing their safety. Recent developments in the field have led to a more refined probiotic selection scheme that consists of five consecutive group tests: stress tolerance, adhesion ability, antipathogenic activity, safety assessment, and clinical trials [35].

3.3. Selection and identification

As noted in the previous subsections, the selection of fructophilic lactic acid bacteria species occurs progressively throughout the isolation and characterization process. The identification of fructophilic lactic acid bacteria candidates is also integral to the process, i.e., identification at the species level is part of the safety assessment step and is achieved by a combination of biochemical assays and 16S rRNA sequencing [35]. At the end of the process, a small number of probiotic candidates is obtained. Based on the highest number of functional properties and absence of negative traits [35], fructophilic lactic acid bacteria species may be selected and regarded as probiotics.

4. Conclusions

Given the unique metabolic capacity of fructophilic lactic acid bacteria and the immense diversity of niches to be explored, it is important to gather information regarding relevant methods for the isolation, characterization, selection, and identification of fructophilic lactic acid bacteria as a guideline for further studies. We hope that the information provided in this mini-review could help accelerate the discovery of fructophilic lactic acid bacteria from fructose-rich environments. The discovery of unique fructophilic lactic acid bacteria species could open opportunities for their application in new fields.

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