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Capillary hydrodynamic chromatography reveals temporal profiles of cell aggregates

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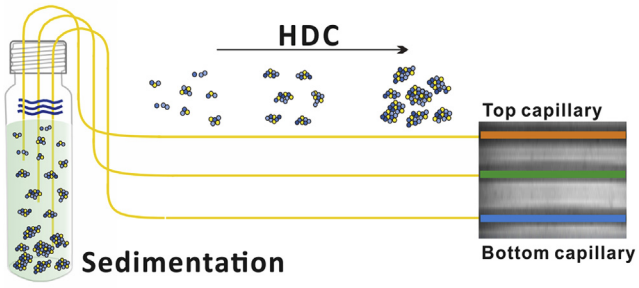
HIGHLIGHTS

- Gravitational sedimentation is coupled on-line with hydrodynamic chromatography.
- Area imaging detection system enables multiplexing hydrodynamic separations.
- On-line and off-line microscopy facilitate peak assignment.
- The approach unravels dynamics of cell aggregates in complex cell suspensions.
- Temporal profiles of microbiomes in probiotic drinks are recorded.

GRAPHICAL ABSTRACT



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ABSTRACT

Microbial cells are known to form aggregates. Such aggregates can be found in various matrices; for example, functional drinks. Capillary hydrodynamic chromatography (HDC) enables separation of particles by size using nanoliter-scale volumes of samples. Here we propose an approach based on HDC for characterisation of real samples containing aggregated and non-aggregated bacterial and fungal cells. Separation of cells and cell aggregates in HDC arises from the parabolic flow profile under laminar flow conditions. In the presented protocol, hydrodynamic separation is coupled with different on-line and off-line detectors (light absorption/scattering and microscopy). The method has successfully been applied in the monitoring of dynamic changes in the microbiome of probiotic drinks. Chromatographic profiles of yogurt and kefir samples obtained at different times during fermentation are in a good agreement with microscopic images. Moreover, thanks to the implementation of an area imaging detector, capillary HDC could be multiplexed and used to profile spatial gradients in cell suspensions, which arise in the course of sedimentation of cells and cell aggregates. This result shows compatibility of sedimentation analysis and capillary HDC. We believe that the approach may find applications in the profiling of functional foods and other matrices containing aggregated bioparticles.

1. Introduction

Probiotic drinks contain live and active cultures of microbial strains along with minerals, vitamins, proteins as well as

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carbohydrates [1–3]. They have been popularised as the prime sources of nutrients that can enrich daily diet, and have beneficial effects on human organism. The microorganisms present in yogurt and other yogurt-like dairy products stay alive in the digestive tract of the host. They crowd out harmful microbes that may cause intestinal infections, they alleviate lactose intolerance or maldigestion [4–6]. Strains that belong to the genera *Lactobacillus* and *Streptococcus* are the most common probiotic microorganisms found in functional foodstuffs [7]. In fact, yogurt is produced by incubating milk with various cultures of bacteria but it does not contain fungal species [8]. Another probiotic drink called "kefir" is produced by adding kefir grains into milk to initiate fermentation. Kefir grains are millimetre-sized, irregular, white-to-yellow particles composed of over 20 different species of bacteria and fungi (yeast) [9,10].

Since probiotic drinks contain numerous microbial species, the cells of those species may interact [11–15]. In general, interactions between different species occur via mechanisms such as amensalism (one species harms another species without being affected itself), competition (species compete for energy sources and nutrients), commensalism (one species benefits while the other species is not harmed), parasitism (one species is benefited but the other species is harmed), and mutualism/symbiosis (two species benefit from the interaction) [10,15]. Notably, production of different kinds of fermented foodstuffs relies on some of these microbial interactions [16]. One such symbiotic interaction between *Streptococcus thermophilus* and *Lactobacillus bulgaricus* leads to an increased growth of microorganisms and accumulation of acid molecules during production of yogurt [10,12,17]. In kefir, yeasts play the key role during fermentation [18,19]. They provide essential growth nutrients such as amino acids and vitamins, alter pH, secrete ethanol and produce carbon dioxide [19]. In one study on kefir granules, it was shown that *Saccharomyces cerevisiae* utilised the lactic acid produced by *Lactobacillus kefiranofaciens* raising pH and stimulating its further growth [12]. Likewise, while studying sourdough fermentation, researchers observed synergy between yeasts and lactic acid bacteria [12].

Biological cells can interact remotely or by direct physical contact. The latter one occurs when cells aggregate [20–26]. Aggregation state of microbial cells is important for the formation of biofilms [24,26], development of antibiotic resistance [27], cell dormancy [25], degradation of organic matter [21], and cell survival [23], to name just a few of several functions. However, formation of cell aggregates is also regarded as a confounding factor in cell-cycle analysis [28]. In fact, cell aggregates may lead to artefacts while conducting flow cytometry [29]. Aggregates can be formed in the course of non-specific interactions between cells or when dividing cells do not separate after cytokinesis. Aggregation of microbial cells in functional drinks may evoke changes in physical and organoleptic properties. For example, it may lead to precipitation of bioparticles in commercial products, thus affecting product shelf life.

In the present study, we propose an approach, based on capillary hydrodynamic chromatography (HDC) [30,31], which enables profiling microbial cell suspensions such as probiotic drinks. In HDC, laminar flow is induced in the separation column by hydrodynamic pumping. Mobile phase molecules attain highest velocity in the centre of the column. Velocity diminishes toward the column wall. Such a flow velocity characteristics is referred to as the parabolic flow profile [32]. Particles present in different regions across the column are exposed to different velocities of the mobile phase. At certain conditions, larger particles may be affected by the flow of the mobile phase to a greater extent than smaller particles [30,31]. Thus, particles with different size can be separated from one another. Here, we utilise this size-dependent separation technique to investigate the dynamics of microbial composition of probiotic drinks. The study was facilitated by different detection techniques, which were coupled with capillary HDC. Moreover, HDC separations were multiplexed in order to combine sedimentation-based fractionation with HDC. This approach is easy to implement – especially when rudimentary screening is sufficient.

2. Experimental section

2.1. Materials and samples

Ammonium acetate and polystyrene monodisperse microparticles (calibrated particle diameters: 20.28 ± 0.02 μm and 8.02 ± 0.10 μm; cat. nos. 87896 and 84192, respectively) were purchased from Sigma–Aldrich (St Louis, MO, USA). Water (LC-MS grade) was purchased from Merck (Darmstadt, Germany). Thiourea was purchased from J.T. Baker (Center Valley, PA, USA). A commercial kefir drink (ingredients: milk, kefir grains: *Lactococcus lactis*, *Lactococcus cremoris*, *Lactococcus diacetylactis*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Saccharomyces lactis*, *Saccharomyces cerevisiae*), a commercial yogurt drink (ingredients: water, milk powder, sucrose, pectin, flavour additive, galactooligosaccharide, *Bifidobacterium lactis* Bb12, *L. acidophilus* La5, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*), and a commercial milk drink (lipids: 3.7 g per 100 mL; proteins: 7.4 g per 100 mL; carbohydrates: 4.8 g per 100 mL; sodium: 42 mg per 100 mL; calcium: 110 mg per 100 mL) were all purchased from local shops (Hsinchu, Taiwan). The original bottles were refrigerated at ~4 °C until the beginning of the experiments. Both artificial sample and probiotic drinks were prepared by mixing the raw material with aqueous solution of ammonium acetate (20 mM, pH ~ 6.0) immediately before the analyses.

2.2. Instrumentation

The capillary HDC experiments were performed by means of the home-made device shown in Fig. 1 and Fig. S1. The HDC injection setup was assembled using inexpensive components, including open-source electronic modules [33]. The sample and solvent were delivered in glass vials (20 mL; cat. no. 88705212140053; Thermo Scientific, Langerwehe, Germany). The solutions were injected hydrodynamically into the fused silica capillary column (75 μm ID, 375 μm OD, 150 cm length; 1010–31,942; GL Science, Tokyo, Japan) by pressurizing gas (injection pressure, 100 kPa) in the headspace of the vial. The pressure inside the vial was monitored by manometer (AZ 8230; AZ Instrument Corporation, Taichung, Taiwan). A pinch valve (18.001.318, nominal voltage 24 V; A.u.K. Müller, Dusseldorf, Germany) was used to control the injection time. It was actuated by a microcontroller (Arduino Pro Micro; Torino, Italy) and a relay (SRD-05VDC-SL-C; Ningbo Songle Relay Company, Yuyao, China). Thanks to the implementation of the Arduino microcontroller, and a custom script, the operation of the system was straightforward and repeatable. When a button on a dial pad was pressed, the valve closed the pressure circuit thus raising the pressure in the headspace of the vial. The valve re-opened after 4 s, terminating hydrodynamic injection. Another button was used to apply the pressure to the mobile phase and to trigger data acquisition by a UV imaging absorption detector (ActiPix D100; Paraytec, York, UK) set to the wavelength of ~200 nm.

2.3. Detection and characterisation

Polystyrene particles and probiotic drinks were prepared in or diluted with ammonium acetate solution (20 mM) before analysis.