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Evaluation of sample extracting methods of FCSM by *Lactobacillus acidophilus* based on a UPLC-Q-TOF-MS global metabolomics analysis

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ABSTRACT

The study of metabolomics requires extracting as many metabolites as possible from a biological sample. This study aimed to determine the optimal method for the extraction of metabolites from solid-state fermented cottonseed meal (FCSM). The UPLC-Q-TOF-MS global metabolomics technology was used to detect the metabolites in FCSM, and the extraction quantity and extraction efficiency of seven different extraction methods, specifically the WA, 50MeOH, 50MeOHB, 50MeCNB, 80MeOHB, 80MeOH and AMF methods were evaluated. The results showed that the number of VIP metabolites extracted by AMF method are 196 and 184 in ESI+ and ESI− mode respectively, it is the largest number of all exacted methods; and the AMF methods also provided a higher extraction efficiency compared with the other methods, especially in indoleacrylic acid, DL-tryptophan and epicatechin ($p < 0.01$). As a result, AMF/−4 °C method was identified as the best method for the extraction of metabolites from FCSM by *Lactobacillus acidophilus*. Our study establishes a technical basis for future metabolomics research of fermented feed.

Introduction

Cottonseed meal (CSM) is the most important plant protein in the world. However, its application in animal husbandry is limited due to its complex content, which includes free gossypol (FG), cyclopropene fatty acids (CPFA) and other anti-nutritional factors, such as a high fibre content, poor protein profile, etc. The most critical problem in CSM is FG, which has a negative effect on animal production.[1–3] Microbial fermentation was used to promote the application and popularization of CSM because it could reduce the FG effectively and improve the nutritional value of CSM, as reported recently.[4–7] *Lactobacillus acidophilus* is an important lactic acid bacteria and intestinal microbe that regulates the intestinal micro-flora balance, enhances immunity, and reduces cholesterol levels.[8–10] But its use for feed fermentation remains rare and few studies have investigated solid-state fermented cottonseed meal (FCSM) by *L. acidophilus*.

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A prerequisite for a metabolomics study of the target metabolites of FCSM by *L. acidophilus* is the optimization of the pretreatment of the biological samples, including scientific sampling, reasonable quenching and high-efficiency extraction.[11,12] The ideal method should produce a sample of all intracellular and extracellular metabolites simultaneously for the analysis of complex biological samples for global metabolomics.[13] Both intracellular and extracellular metabolites exert a significant effect on the results of microbial metabolomic studies.[14] Fermented feed is a complex biological sample; the fermentation products not only include the metabolites of microbial fermentation but also include the fermentation substrate nutrients. To truly reflect the fermentation process, the pretreatment methods of quenching and extracting metabolites must be optimized.

Previous studies of the pretreatment of biological samples have demonstrated that different quenching and extracting methods were suitable for different microorganisms, such as *Escherichia coli*, *Yeast*, *Lactic acid bacteria*, etc.[15–19] These studies mainly included extracting microorganism metabolites by screening different pretreatment methods and examining the effects of different quenching solvents on the extraction of metabolites and sample grinding degree. However, these evaluations studied how to use different research platforms and indicators of the extraction method. The majority of studies were of the extraction of different quenching solvents, with a research platform based on traditional GC–MS and LC–MS. Recent developments have proved that UPLC-Q-TOF-MS could detect complex multi-component substances more effectively, faster and more accurately than other methods.[20–22] At the same time, the UPLC-Q-TOF-MS platform can also be analysed quantitatively for the number of metabolites. So far, a few applications of *L. acidophilus* to metabolomics have been studied. Since *L. acidophilus* fermented protein feed is rare for traditional feed production, the method of extracting metabolites from FCSM has not received sufficient attention. Therefore, it was desirable to establish an extraction method that can be applied to a wide range of metabolites from *L. acidophilus*.

To identify the final metabolites in FCSM by *L. acidophilus*, both intracellular and extracellular metabolites must be analysed. We used the global metabolomics profiling way to evaluate the efficiency of seven extraction methods (WA, 50MeOH, 50MeOHB, 50MeCNB, 80MeOHB, 80MeOH and AMF) systematically with the aim of identifying the best method for the extraction from the FCSM. Seven different pretreatment methods were evaluated through a global metabolomics analysis based on UPLC-Q-TOF-MS. The quantity and efficiency of the metabolites extracted from FCSM were evaluated. This study will provide a good basis for future analyses of the total metabolites in the FCSM.

Materials and methods

Chemicals

LC–MS-grade methanol and acetonitrile were purchased from Fisher Chemical (Fisher Scientific, USA) or Sigma–Aldrich (St. Louis, MO, USA). The water was purified with an EPED-E2-T device (Nanjing, China).

Sample preparation

L. acidophilus (JJ-0787) was acquired from the China Center of Industrial Culture Collection (CICC). Fermentation substrates were composed of 65.89% CSM and 34.11% corn, with a total protein of 30%. JJ-0787 strains were cultured at 37 °C for 24 h in MRS broth (Solarbio Life Sciences Ltd., Beijing, China), and then inoculated 6 mL JJ-0787 strains culture fluid in 100 g sterilized fermentation substrate (the moisture content is 40%), mixed thoroughly, sealed in the triangle bottle, and then statically placed in an incubator at 37 °C for 48 h. Each group contained three repetitions. The resulting samples of FCSM were immediately frozen in liquid nitrogen and then stored at −80 °C until analysis.

Metabolite extraction

To investigate the optimal method for the extraction of the metabolites in FCSM, the different quenching solvents and pretreatment methods used in the studies conducted by Tokuoka and Chen were modified as follows.[18,19]

WA (J1 group): A sample of FCSM was treated with high-speed homogenization (HHM). Then, a 0.2-g sample was rapidly quenched and dissolved in 2 mL of ultra-pure water (4 °C). The samples were quickly homogenized for 10 min using a high-flux tissue homogenizer (TisssueLyser II, Qiagen, Germany). The centrifuge tubes were incubated for 30 min on ice. The extract solution was centrifuged (−20 °C, 10,000 r/min, 5 min), and the residual debris was removed. The supernatant was dissolved in 10 volumes of ultra pure water (4 °C) and filtered with a 0.22-μm syringe filter (004022NL-SEPTFE, Shanghai, China). The samples were stored at −80 °C until further analysis.

50MeOH (J2 group): The extraction process was the same as that used in the WA method except that the quenching solvent was replaced by a 50% cold methanol solution (methanol:water = 50:50, −20 °C).

50MeOHB (J3 group): The extraction process was identical to that used in 50MeOH method except that the 50% cold methanol solution (−20 °C) was replaced with a 50% cold methanol solution (room temperature), and after the addition of quenching solvent to the sample, the method incorporated a boiling procedure (70 °C, 5 min) immediately followed by the same process as used in 50MeOH method.

50MeCNB (J4 group): The extraction procedures were identical to those used in 50MeOHB method with the exception that the 50% methanol solution (room temperature) was replaced by a 50% acetonitrile solution (acetonitrile:water = 50:50, room temperature).

80MeOHB (J5 group): The 50% methanol solution used in 50MeOHB method was replaced with a 80% methanol solution (methanol:water = 80:20, room temperature), followed by the same method as in 50MeOHB.

80MeOH (J6 Group): The extraction procedures were identical to those used in 50MeOH method except that the 50% cold

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