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Rapid Detection of *Bifidobacterium bifidum* in Feces Sample by Highly Sensitive Quartz Crystal Microbalance Immunosensor

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In this work, a quartz crystal microbalance (QCM) sensor has been fabricated using immunoassay for sensitive determination of *Bifidobacterium bifidum*. Au nanoparticle has been used for amplifying sandwich assays. The proposed immunosensor exhibited a linear detection range between 10^3 and 10^5 CFU/mL with a limit of detection of 2.1 × 10^2 CFU/mL. The proposed immunosensor exhibited good selectivity for *B. bifidum* sensing with low cross reactivity for other foodborne pathogens such as *Lactobacillus acidophilus*, *Listeria monocytogenes*, and *Escherichia coli*. In addition, the proposed immunosensor has been successfully used for *B. bifidum* detection in feces samples and food samples. The frequency decreases of 12, 17, and 10 Hz were observed from the milk samples consisting of the mixtures of *L. acidophilus*, *L. monocytogenes*, and *E. coli*. The frequency decreases of 8, 15, and 7 Hz were observed from the feces samples consisting of the mixtures of *L. acidophilus*, *L. monocytogenes*, and *E. coli*.

Keywords: quartz crystal microbalance sensor, *Bifidobacterium bifidum*, immunosensor, Au nanoparticle, feces sample

INTRODUCTION

Under normal circumstances, the intestinal microorganisms in the human body form a relatively balanced state. Once the balance is damaged, it will lead to the imbalance of intestinal flora (Horie et al., 2017; Xue et al., 2018). Some intestinal microorganisms, such as *Clostridium perfringens*, overproduce in the intestinal tract and produce harmful substances such as ammonia, amines, hydrogen sulfide, manure, indole, nitrite, and bacterial toxins, which will further affect the health of the body (Ashida et al., 2018; Duranti et al., 2019). *Bifidobacterium bifidum* is an important intestinal beneficial microorganism. *B. bifidum*, as a physiological beneficial bacterium, has many important physiological functions for human health, such as biological barrier, anti-tumor, immune enhancement, improvement of gastrointestinal function, and anti-aging (Wang et al., 2016; Mauras et al., 2018; Din et al., 2020). *B. bifidum* can inhibit the growth of harmful bacteria, resist the infection of pathogenic bacteria, and synthesize vitamins needed by human body. It can promote

the absorption of minerals, produce organic acids such as acetic acid, propionic acid, butyric acid, and lactic acid to stimulate intestinal peristalsis and promote defecation. It can prevent constipation, purify the intestinal environment, decompose carcinogens, stimulate the human immune system, and improve the disease resistance (Gomi et al., 2018; Bondue et al., 2019; Speciale et al., 2019). It is of crucial importance to identify *B. bifidum* either in food and excreta. Although the normal microbiological isolation methods have been widely used for identifying bacterial strains, they are time-consuming (Yang et al., 2017; Cheng et al., 2018; Faraki et al., 2020). Therefore, the development of a fast method for *B. bifidum* identification is very essential for sample scanning.

The biosensor first immobilizes the bioactive components (enzyme, antibody, tissue, cell) on the transducer. When the target analyte is recognized by the immobilized bioactive components, the biochemical reaction can be immediately converted into a quantifiable electrical signal through the transducer (Fu et al., 2015; Huertas et al., 2019; Alamgholiloo et al., 2020; Fouladgar et al., 2020; Karimi-Maleh et al., 2020). Since the rise of biosensor in the late 1960s, after nearly half a century of development, biosensor has become a comprehensive and interdisciplinary field, which is used in food safety testing, environmental testing, and clinical diagnosis. QCM is a new type of micro mass sensor based on quartz crystal resonance, which was developed in 1960s. According to the piezoelectric effect of quartz crystal, the resonance frequency of quartz crystal will change with the mass of adsorbed material, and they are in a positive proportion (Bearzotti et al., 2017; Speller et al., 2017; Wang A. et al., 2017; Ayankojo et al., 2018). QCM immunosensor is a specific biosensor combining the high sensitivity of quartz crystal and the high specificity of immune response (Bearzotti et al., 2017; Speller et al., 2017; Wang L. et al., 2017; Ayankojo et al., 2018; Zhang et al., 2018). At present, QCM immunosensor has been widely used in clinical testing, food hygiene, environmental testing, as well as other fields of chemical analysis, and biological analysis. The detection of bacteria by QCM is a new and attractive Research Topic in electronic informatics and medicine (Muckley et al., 2016; Tai et al., 2016; Chen et al., 2018; Ding et al., 2018; Lal and Tiwari, 2018; Temel et al., 2019).

In this work, a QCM immunosensor has been developed for sensitive determination of *B. bifidum*. Monoclonal and polyclonal antibody have been used with the signal amplifying based on the Au nanoparticle. The proposed immunosensor exhibited a wide linear detection range with a low detection of limit. The sensitivity was enhanced when the antibody-conjugated Au nanoparticle. We believe the proposed assay was validated by cross reactivity investigation using *Lactobacillus acidophilus*, *Listeria monocytogenes* and *Escherichia coli*.

MATERIALS AND METHODS

Reagents, Bacterial Strains, and Instruments

Polyclonal antibody and mouse monoclonal antibody against *B. bifidum* were purchased from San Ying Biotechnology Co., Ltd (Wuhan, China). Mouse IgG, 11-mercaptoundecanoic acid (11-MUDA), bovine serum albumin (BSA), and ethanolamine hydrochloride were purchased from Linc-Bio Science Co., Ltd (Shanghai, China). Au colloidal (AuNPs) with 20 nm was purchased from Shenzhen Nano Tech Co., Ltd (Shenzhen, China). All other common regents were supplied by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) and used without further purification. A QCA922 quartz crystal analyzer (Princeton, USA) has been used for sensing with an Au coated chips. *Bifidobacterium bifidum* (*B. bifidum*), *L. acidophilus*, *L. monocytogenes*, and *E. coli* strains were purchased from American Type Culture Collection (ATCC, Manassas, VA). MRS (de Man, Rogosa, and Sharpe) and Bifidus Selective Medium (BSM) were used as growth media.

Sensor Chip Preparation

Piranha solution has been used for removing any impurities on the QCM Au chip. Then, the Au chip was immersed into 20 mM thiol (11-MUDA, prepared using ethanol) overnight for fabricating carboxy-terminated thiol layer on the Au surface. After rinse by ethanol and water, the Au chip was used for baseline correction under 10 mM PBS (pH 7.4). A mixture solution of EDC (0.4 M)-NHS (0.1 M) at ratio of 1:1 was then used for sensor activation. Then, different concentrations of polyclonal or mouse monoclonal antibodies against *B. bifidum* were immobilized on the Au chip. Then, capture and control were carried out by injected either polyclonal or mouse monoclonal antibodies and mouse IgG antibody. Then, BSA (50 μg/mL) and ethanolamine (1 M) were used for blocking and capping the sensors.

Detection of *B. bifidum*

Direct detection of *B. bifidum* was carried out by the injection of different concentrations of *B. bifidum* cells suspension prepared in PBS over above-mentioned polyclonal, mouse monoclonal, and mouse IgG antibodies fabricated sensor. The detection was carried out by comparing either *B. bifidum* captured polyclonal or mouse monoclonal antibodies with the control group.

The sandwich assay with antibody-AuNPs conjugation was prepared according to previous methods with some modifications (Uludag and Tothill, 2012). Typically, 0.1 mL of polyclonal or mouse monoclonal antibodies was added into 0.5 mL of AuNPs suspension with 2 h slow stirring. Then, 0.1 mL of BSA (10%) was introduced to the mixture with 2 h slow stirring. A centrifugation process was carried out to remove the excess of BSA and AuNPs. The detection process of using antibody-AuNPs conjugation was similar to the direct detection except the polyclonal and mouse monoclonal antibodies were replaced by the polyclonal-AuNPs and mouse monoclonal-AuNPs antibodies. Non-linear regression with four parameter logistic equations has been used for construing the calibration curves (Karpinski, 1990).

Selectivity Test

L. acidophilus, *L. monocytogenes*, and *E. coli* have been used for evaluating the specificity of the assay to *B. bifidum*. The selectivity test only carried out using antibody-AuNPs conjugation. The detection process of

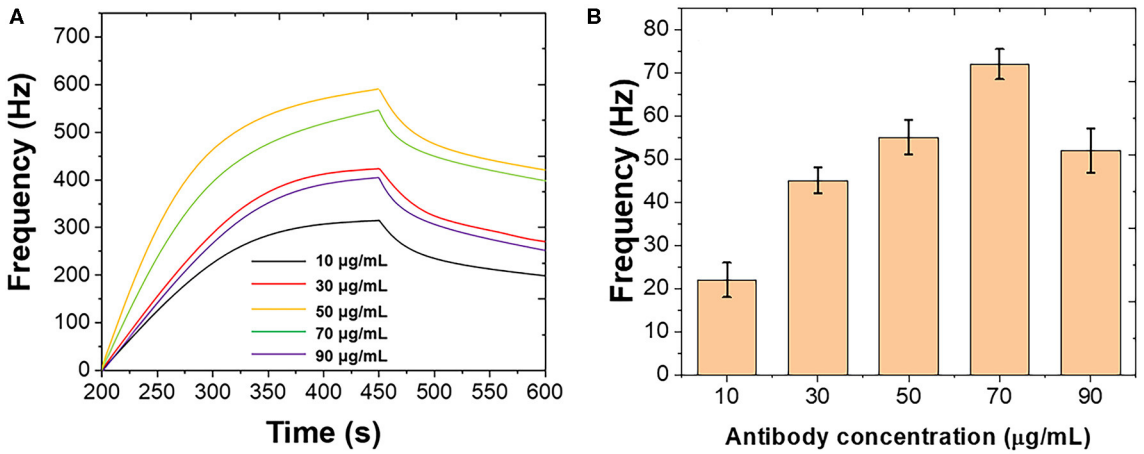

FIGURE 1 | (A) Frequencies recorded at QCM when different concentrations of polyclonal antibody immobilized on the Au chip surface. **(B)** Frequencies performance of QCM after introduction of *B. bifidum* after the immobilization of different concentrations of polyclonal antibody.

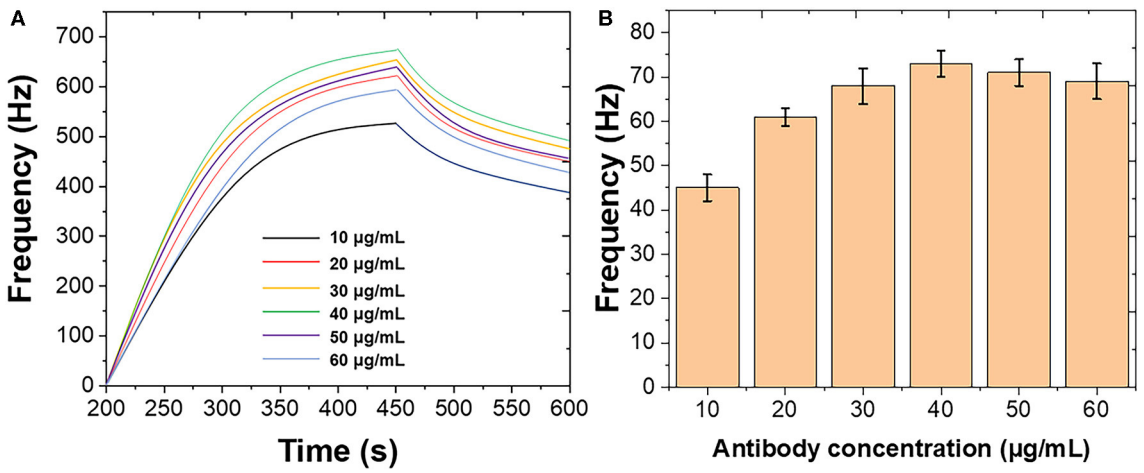

FIGURE 2 | (A) Frequencies recorded at QCM when different concentrations of mouse monoclonal antibody immobilized on the Au chip surface. **(B)** Frequencies performance of QCM after introduction of *B. bifidum* after the immobilization of different concentrations of mouse monoclonal antibody.

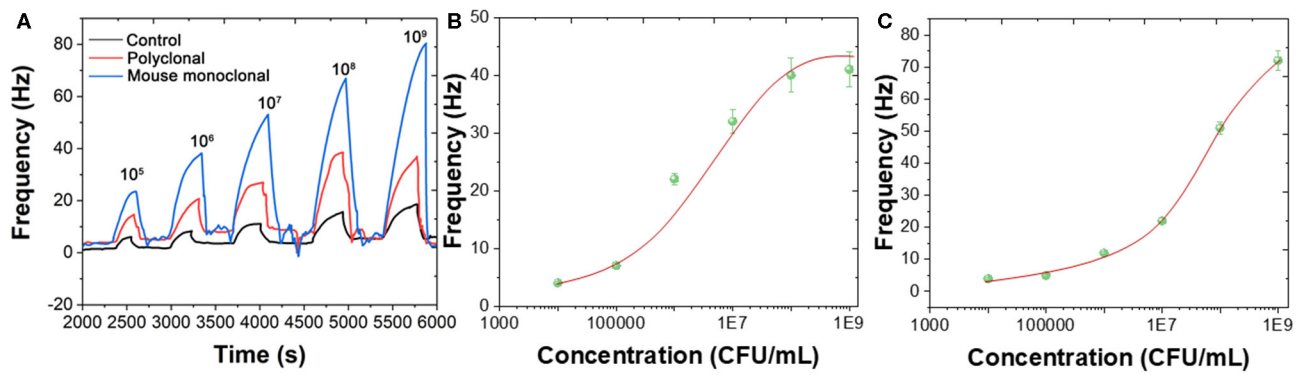

FIGURE 3 | (A) Frequencies recorded at QCM when different concentrations of *B. bifidum* introduced using polyclonal antibody immobilized sensor, mouse monoclonal antibody immobilized sensor, and mouse IgG antibody immobilized sensor. Plots of Frequency vs. concentrations of *B. bifidum* using **(B)** polyclonal antibody immobilized sensor and **(C)** mouse monoclonal antibody immobilized sensor.

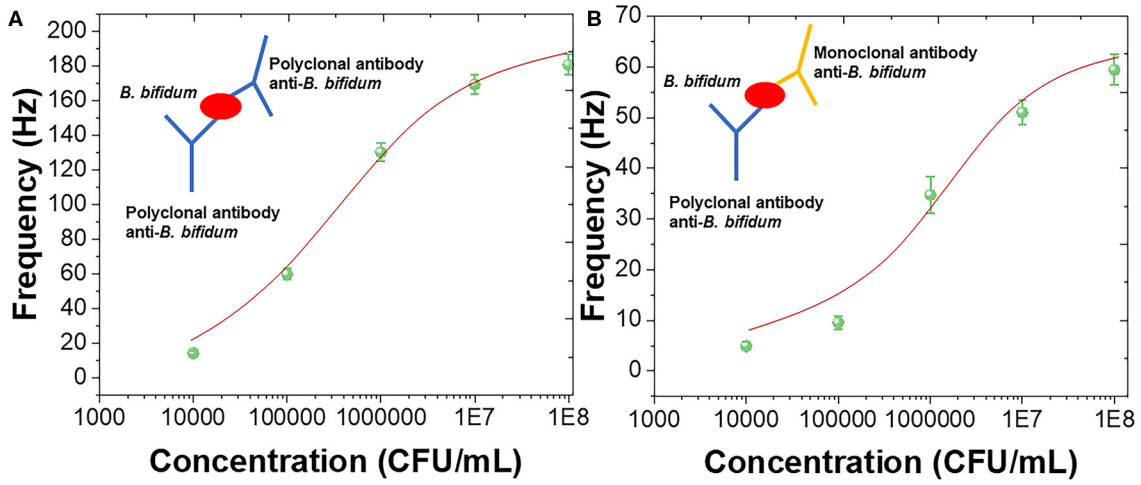

FIGURE 4 | Frequency vs. concentrations of *B. bifidum* using **(A)** polyclonal antibody and **(B)** mouse monoclonal antibody as the detection antibodies.

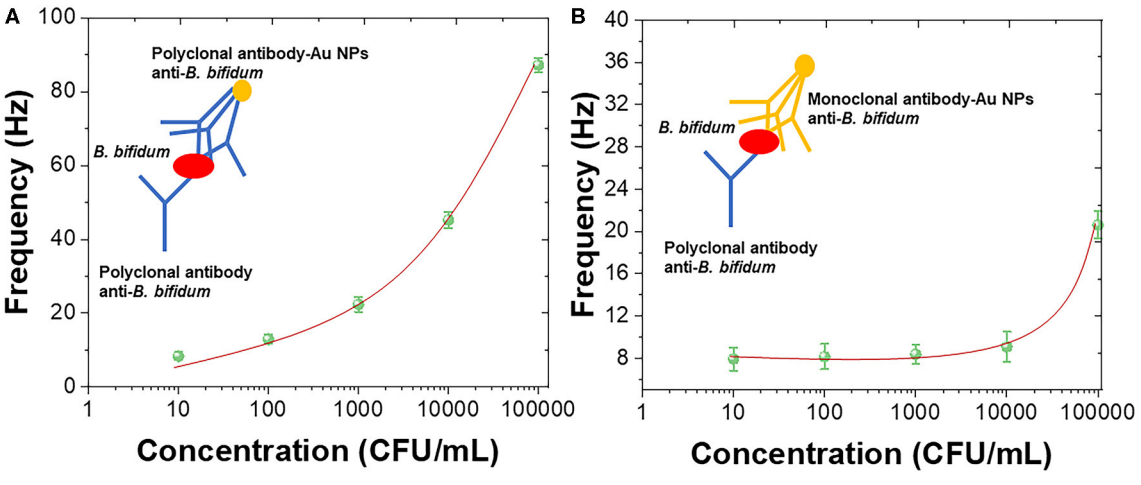

FIGURE 5 | Frequency vs. concentrations of *B. bifidum* using **(A)** polyclonal antibody-AuNPs conjugation and **(B)** mouse monoclonal antibody-AuNPs conjugation as the detection layers.

was similar to the above-mentioned protocol except replaced *B. bifidum* by *L. acidophilus*, *L. monocytogenes*, and *E. coli*.

Real Sample Preparation

The sensing of *B. bifidum* in milk and feces were investigated. Fresh milk was purchased form local supermarket. Feces samples were provided by The First Affiliated Hospital of Shantou University Medical College. For the sensing of *B. bifidum*, 1 g of real sample was dispersed using 50 mL PBS and transferred into a filtering stomacher bag. Then, 0.5 mL of the *B. bifidum* suspension was inoculated into both real samples at 37°C for 2 h. All liquid samples after preparation were directly used for sensing purpose.

RESULTS AND DISCUSSION

Figure 1 shows the optimization of the immobilization of polyclonal antibody on the Au chip surface. Different concentrations of polyclonal antibody were introduced using the standard *B. bifidum*. As shown in **Figure 1A**, the frequency was initially increased when the polyclonal antibody increased from 10 to 50 μg/mL. Then, the frequency decreased when the further increase of the polyclonal antibody. **Figure 1B** shows the binding response of *B. bifidum* at these concentrations of polyclonal antibody used. It can be seen that the best response was achieved when the concentration of polyclonal antibody at 50 μg/mL. Therefore, 50 μg/mL of polyclonal antibody has been selected in this study.

Figure 2 shows the optimization of the immobilization of mouse monoclonal antibody on the Au chip surface. Different

TABLE 1 | Sensing performance of the proposed QCM sensor with other reports.

Analyte	Linear detection range	Limit of detection	References
S. paratyphi	10^2-10^5 CFU/mL	1.7×10^2 CFU/mL	Fung and Wong, 2001
E. coli O157:H7	0–1 log CFU/mL	–	Guo et al., 2012
α-Amylase	–	1 μg/mL	Della Ventura et al., 2017
H5N1 avian influenza virus	2^{-4}-2^4 HAUs/50 μL	2^{-4} HAU/50 μL	Wang L. et al., 2017
Albumin	35–55 mg/mL	0.234 mg/mL	Pohanka, 2018a
S. aureus	–	5.18×10^8 CFU/mL	Pohanka, 2020
B. bifidum	10^3-10^5 CFU/mL	2.1×10^2 CFU/mL	This work

concentrations of mouse monoclonal antibody were introduced using the standard *B. bifidum*. As shown in **Figure 2A**, the frequency was initially increased when the mouse monoclonal antibody increased from 10 to 40 μg/mL. Then, the frequency decreased when the further increase of the mouse monoclonal antibody. **Figure 2B** shows the binding response of *B. bifidum* at these concentrations of mouse monoclonal antibody used. It can be seen that the best response was achieved when the concentration of mouse monoclonal antibody at 50 μg/mL. Therefore, 50 μg/mL of mouse monoclonal antibody has been selected in this study. Both studies suggested a high concentration of antibodies could result in a high steric hindrance, which lower the sensitivity of the sensor. Similar results were obtained for *Campylobacter jejuni* and *Vibrio harveyi* as well (Buchatip et al., 2010; Masdor et al., 2016).

B. bifidum can be directed detected using both polyclonal and mouse monoclonal antibodies immobilized Au chips. **Figure 3** shows the frequency changes when different concentrations of *B. bifidum* introduced in the QCM system in the presence of polyclonal antibody immobilized sensor, mouse monoclonal antibody immobilized sensor, and mouse IgG antibody immobilized sensor. As shown in **Figures 3B,C**, sigmoidal relationship between frequency change signals and concentrations of *B. bifidum* were obtained for both polyclonal antibody immobilized sensor and mouse monoclonal antibody immobilized sensor. The linear portions of the concentrations of *B. bifidum* between 10^5-10^7 and 10^6-10^8 CFU/mL were obtained for polyclonal antibody immobilized sensor and mouse monoclonal antibody immobilized sensor, respectively. The limit of detection (LOD) of polyclonal antibody immobilized sensor and mouse monoclonal antibody immobilized sensor can be calculated to be 3.0×10^5 and 3.0×10^6 CFU/mL, respectively. Since the polyclonal antibody immobilized sensor exhibited a lower LOD, it has been further used for construction of sandwich assay.

The sandwich assay has been constructed using polyclonal antibody against *B. bifidum*. The sandwich assay could improve sensitivity as well as prevented the false negative results (Amani et al., 2018). **Figure 4A** shows the sensing performance of using polyclonal antibody as the capture and detection antibody with schematic diagram. **Figure 4B** shows the sensing performance of using polyclonal antibody as the capture and mouse monoclonal antibody as the detection antibody with schematic diagram. It can be seen that both sandwich assay exhibited sigmoidal relationship between frequency and concentrations of *B. bifidum*. Linear portions of the concentrations of *B. bifidum* between 10^4-10^8 and 10^5-10^8 CFU/mL were observed using polyclonal antibody and mouse monoclonal antibody as the detection antibodies, respectively. The LOD of using polyclonal antibody and mouse monoclonal antibody as the detection antibodies can be calculated to be 2.0×10^4 and 3.3×10^5 CFU/mL, respectively. These results indicated that the sandwich assay could increase the sensitivity of the QCM sensor (Cervera-Chiner et al., 2018; Makhneva et al., 2018; Pohanka, 2020).

In order to further enhance the sensitivity of the QCM immunosensor, Au nanoparticles were introduced for forming the antibody-AuNPs conjugation (Zheng et al., 2015; Wang L. et al., 2017; Fu et al., 2020; Xu et al., 2020). **Figure 5A** shows the sensing performance of using polyclonal antibody-AuNPs conjugation as detection layer with schematic diagram. **Figure 5B** shows the sensing performance of using mouse monoclonal antibody-AuNPs conjugation as detection layer with schematic diagram. A linear portions of the concentrations of *B. bifidum* between 10^3 and 10^5 CFU/mL was observed using polyclonal antibody-AuNPs conjugation as detection layer. The LOD of using mouse monoclonal antibody-AuNPs conjugation can be calculated to be 2.1×10^2 CFU/mL. No clear linear portions of the concentrations of *B. bifidum* can be observed using mouse monoclonal antibody-AuNPs conjugation as detection layer. The results recorded in this study are very competitive with previous reports (Fung and Wong, 2001; Guo et al., 2012; Skládal, 2016; Della Ventura et al., 2017; Wang R. et al., 2017; Gao et al., 2018; Pohanka, 2018a,b, 2019). **Table 1** showed the sensing performance comparison.

The selectivity of the proposed QCM immunosensor has been tested by *L. acidophilus*, *L. monocytogenes*, and *E. coli*. As shown in **Figure 6**, <10% of cross reactivities were observed by *L. acidophilus*, *L. monocytogenes*, and *E. coli*, suggesting the proposed QCM immunosensor exhibited an excellent selectivity toward *B. bifidum*.

The applicability of the novel QCM immunosensor to determine *B. bifidum* in milk and feces were also carried out. For the detection of *B. bifidum* in real samples, the QCM sensor was firstly calibrated by the measurement in the absence of *B. bifidum*. As shown in **Figure 7**, frequency change of 3 and 7 Hz were recorded from the milk sample and feces sample

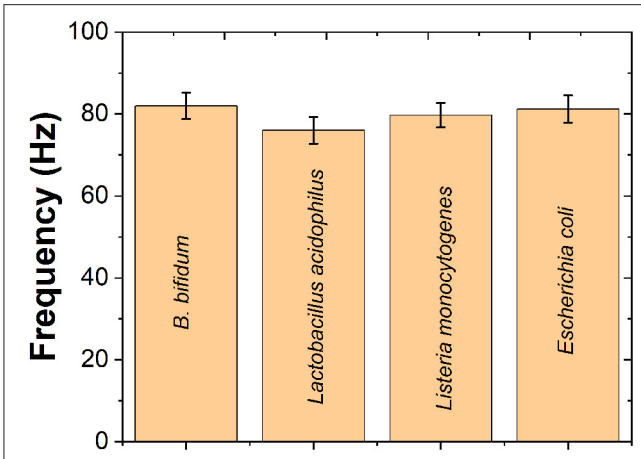

FIGURE 6 | Selectivity performance of the proposed QCM immunosensor toward *B. bifidum* sensing compared with that of the *Lactobacillus acidophilus*, *Listeria monocytogenes* and *Escherichia coli*.

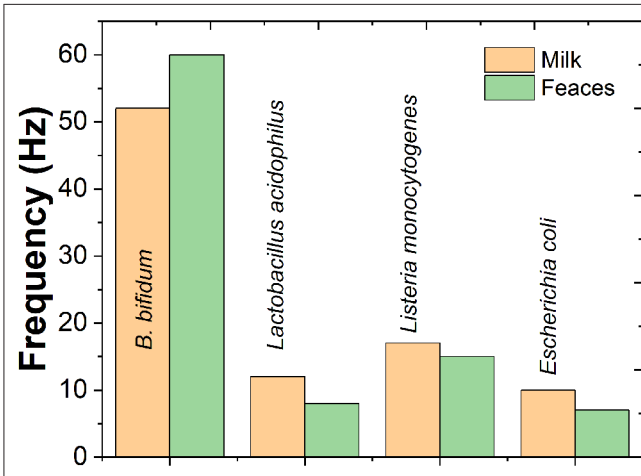

FIGURE 7 | Specific detection of 10^4 CFU/mL of *B. bifidum*, *Lactobacillus acidophilus*, *Listeria monocytogenes* and *Escherichia coli* inoculated on milk and feces samples.

containing 10^4 CFU/mL of *B. bifidum*, respectively. These results suggested the proposed QCM immunosensor could be used for real sample test. In addition, the frequency decreases of 12, 17, and 10 Hz were observed from the milk samples consisting of the mixtures of *L. acidophilus*, *L. monocytogenes*, and *E. coli*. The frequency decreases of 8, 15, and 7 Hz were observed from the feces samples consisting of the mixtures of *L. acidophilus*, *L. monocytogenes*, and *E. coli*. The results indicate that the QCM system developed in the present study is practical for the simultaneous enrichment and detection of viable *B. bifidum* in real samples.

CONCLUSION

In this work, an advanced piezoelectric biosensor-QCM system was proposed. In order to further enhance the sensitivity of the QCM immunosensor, Au nanoparticles were introduced for forming the antibody-AuNPs conjugation. A linear portions of the concentrations of *B. bifidum* between 10^3 and 10^5 CFU/mL was observed using polyclonal antibody-AuNPs conjugation as detection layer. The LOD of using mouse monoclonal antibody-AuNPs conjugation can be calculated to be 2.1×10^2 CFU/mL. The selectivity of the proposed QCM immunosensor has been tested by *L. acidophilus*, *L. monocytogenes*, and *E. coli*. The applicability of the novel QCM immunosensor to determine *B. bifidum* in milk and feces were tested. The frequency decreases of 12, 17, and 10 Hz were observed from the milk samples consisting of the mixtures of *L. acidophilus*, *L. monocytogenes*, and *E. coli*. The frequency decreases of 8, 15, and 7 Hz were observed from the feces samples consisting of the mixtures of *L. acidophilus*, *L. monocytogenes*, and *E. coli*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

KH, YC, and JL contributed conception and design of the study. GL, YL, and DC conducted QCM experiments. DZ and ZW performed the statistical analysis. DL and XH did the characterizations. KH and YC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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