Development of a PDMS/paper hybrid microfluidic device in investigating antimicrobial susceptibility of pathogenic bacteria in poultry products

Xinyu Yan

Department of Food Science and Agricultural Chemistry

Faculty of Agricultural and Environmental Sciences

McGill University, Montreal

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ABSTRACT

Antimicrobial resistance (AMR) in foodborne pathogens has been a continuous concern and a serious threat to public health. The ascending resistance makes the clinical treatment more challenging to take effect on adverse health manifestations and leads to prolonged recovery periods and high mortality rates. Therefore, a rapid, portable, and easy-to-operate device needs to be developed for detection of AMR bacteria in food products. In this thesis project, a PDMS/paper hybrid microfluidic chip based on colorimetric reaction was developed to carry out both identification and antimicrobial susceptibility test (AST) simultaneously. This microfluidic device is prominent due to its cost-effective nature, high efficiency, and compatibility to point-of-care diagnostics. *Campylobacter* and *Salmonella* were selected as the target bacteria and cultivated on chromogenic agar loaded in the microfluidic chamber with the addition of certain antibiotics. This microfluidic device showed a limit of detection (LOD) of 10^2 CFU/mL using pure bacterial culture. In comparison, the LOD was 10^4 CFU/25 g spiked poultry samples. The categorical agreement rate between the on-chip AST and conventional broth dilution method was over 90%. The outcome of this thesis project showed the feasibility of applying this microfluidic device by the agri-food industry to prevent the AMR crisis in an early stage.

RÉSUMÉ

La résistance aux antimicrobiens (RAM) des agents pathogènes d'origine alimentaire est une préoccupation constante et une menace sérieuse pour la santé publique. La résistance croissante rend le traitement clinique plus difficile à prendre en compte sur les manifestations indésirables de la santé et conduit à des périodes de récupération prolongées et à des taux de mortalité élevés. Par conséquent, un dispositif rapide, portable et facile à utiliser doit être développé pour la détection des bactéries RAM dans les produits alimentaires. Dans ce projet de thèse, une puce microfluidique hybride PDMS/papier basée sur une réaction colorimétrique a été développée pour effectuer simultanément l'identification et le test de sensibilité aux antimicrobiens (AST). Ce dispositif microfluidique est important en raison de sa nature rentable, de son efficacité élevée et de sa compatibilité avec les diagnostics au point de service. *Campylobacter* et *Salmonella* ont été sélectionnés comme bactéries cibles et cultivés sur gélose chromogénique chargée dans la chambre microfluidique avec l'ajout de certains antibiotiques. Ce dispositif microfluidique a montré une limite de détection (LOD) de 10^2 UFC/mL en utilisant une culture bactérienne pure. En comparaison, la limite de détection était de 10^4 UFC/25 g d'échantillons de volaille dopés. Le taux de concordance catégorique entre l'AST sur puce et la méthode conventionnelle de dilution en bouillon était supérieur à 90 %. Les résultats de ce projet de thèse ont montré la faisabilité de l'application de ce dispositif microfluidique par l'industrie agroalimentaire pour prévenir la crise de la RAM à un stade précoce.

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PREFACE

This thesis was prepared by following the thesis guidelines for graduate and postdoctoral studies from McGill University. There are five chapters included in this thesis, namely Introduction, Literature Review, Materials and Methods, Results and Discussion, and Conclusion. This work is original and has not been published previously.

CONTRIBUTION OF AUTHORS

Xinyu Yan is the first author who is responsible for the entire thesis. Dr. Xiaonan Lu proposed this thesis project, and Dr. Luyao Ma had a direct advisory on all the experimental plans. Xinyu Yan performed all the experiments, including the design and fabrication of the microfluidic device, optimization of chip performance, on-chip identification and antimicrobial susceptibility test, on-chip tests using spiked food samples, and data analysis. This thesis was edited and guided by Dr. Lu and Dr. Ma.

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LIST OF ABBREVIATIONS

amp	ampicillin
AMR	Antimicrobial resistance
AST	antimicrobial susceptibility testing
AuNP	gold nanoparticle
Cip	ciprofloxacin
CLSI	Clinical Laboratory Standard Institute
DNA	deoxyribonucleic acid
DPV	differential pulse voltammetry
ery	erythromycin
FQ	fluoroquinolones
GBS	Guillain-Barré syndrome
GOx	Glucose oxidase
GO	graphene oxide
LAMP	loop mediated isothermal amplification
LB	Luria Bertani
LED	light-emitting diode
LOC	Lab-on-chip
LOD	limit of detection
LOQ	limit of quantification
MDR	multidrug resistance
MFS	Miller Fisher syndrome
MH	Müller Hinton
MIC	minimum inhibitory concentration
mRNA	messenger ribonucleic acid
NAHMS	National Animal Health Monitoring System
NC	negative control
PC	polycarbonate
PCR	polymerase chain reaction
PDMS	polydimethylsiloxane

PET	polyethylene terephthalate
PMA	propidium monoazide
PMMA	poly(methyl methacrylate)
POC	point-of-care
PS	polystyrene
PVC	polyvinyl chloride
QDs	quantum dots
RNA	ribonucleic acid
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SERS	surface-enhanced Raman spectroscopy
SO_2	sulfur dioxide
tet	tetracycline
VBNC	viable-but-non-culturable

CHAPTER 1. INTRODUCTION

1.1 General Introduction

Antimicrobial resistance (AMR) of bacteria demonstrates that bacteria cannot be inhibited by antibiotics, which leads to limited treatment options. AMR crisis is associated with millions of infected cases and thousands of deaths. There are even more bacteria that become resistant every day while current treatments still heavily depend on antibiotic usage (Goedhart et al., 2014; Murray et al., 2022). As a consequence, the AMR crisis can cause failed treatments, extended recovery, and increasing mortality rate (Toner et al., 2015). To prevent this type of crisis, early detection is a feasible way. The conventional technologies for detecting AMR [e.g., antimicrobial susceptibility testing (AST)] are generally based on central laboratories. They are usually time-consuming, equipment-dependent, and labor-intensive (Aroonnual et al., 2017; Lazcka et al., 2007). Other alternative techniques such as polymerase chain reaction (PCR), genome sequencing, and mass spectrometry are applied to address these drawbacks. However, these advanced techniques still cannot sufficiently provide well-rounded benefits, including rapid process and cost-effective procedures (Hrabák et al., 2013; Pulido et al., 2013; Schofield, 2012).

Microfluidic “lab-on-a-chip” is a technique that can be used in different applications, such as food analysis, clinical diagnostics, and environmental monitoring (Sridhar et al., 2021). It provides numerous advantages over the traditional macro-scale methods. With a small dimension in the microfluidic system, cells or molecules can have reduced diffusion distance. As a result, the process in the microfluidic devices is more rapid (Convery & Gadegaard, 2019). The characteristics of miniaturization can effectively reduce the fabrication cost and enhance the portability that is suitable for point-of-care diagnostics.

Microfluidic-based bacterial detection can be divided into two categories, namely genotypic and phenotypic sensing. Genotypic sensing aims at specific genetic markers that can provide detailed information about the bacterial species (Wang et al., 2011; Zhang et al., 2018). However, it cannot always determine the antimicrobial susceptibility profile without the prior knowledge of antimicrobial resistant mechanisms (Tang et al., 2017). Phenotypic sensing targets monitoring the growth of bacteria with the presence of antibiotics. It usually confines bacteria in a small system, such as channels and chambers (Hassan & Zhang, 2020; Matsumoto et al., 2016). Bacteria are often selectively captured by antibodies that are immobilized on the surface of the

channels or chambers within the microfluidic device (Dong & Zhao, 2015; J. He et al., 2014). Bacterial growth can then be monitored by using an external equipment, such as a microscope and a Raman spectrometer (Lu et al., 2013; Pitruzzello et al., 2019) that are not affordable in all the fields.

To eliminate the involvement of an external detector, the microfluidic "lab-on-a-chip" platform has been designed based on colorimetric reaction using the chromogenic agar. Several previous studies validated the effectiveness of this concept and design. For example, Iseri and others developed a digital dipstick loaded with chromogenic agar for quantitative detection of *Escherichia coli* (Iseri et al., 2020). In another study, Wen and others developed a microfluidic device that could achieve multiplex detection of four types of bacteria simultaneously on the basis of colorimetric reaction (Wen et al., 2014). However, these studies only focused on bacterial identification, but did not carry out AST. Regarding AST, Cira and others developed a device that could determine the antimicrobial susceptibility of pathogens via the colorimetric change of pH indicator due to bacterial growth (Cira et al., 2012). This method had a potential limitation towards those acid-produced bacterial species since the normal metabolism may lead to a pH change and consequently a false positive result (Reis et al., 2012). In another study conducted by Elavarasan and co-authors, resazurin dye reduction (i.e., intense blue color changing to pink and leuco) was applied to visualize the growth of bacteria with antibiotics (Elavarasan et al., 2013).

To perform bacterial identification test and AST simultaneously, chromogenic agar with the addition of antibiotics can be applied to allow the target bacteria to selectively grow in an enclosed envrionemnt. A previous study by Xu and others applied this method to identify urinary tract infection pathogens and carried out AST by using chromogenic agar (Xu et al., 2016). In another study, Ma and co-authors developed a chromogenic agar-based microfluidic "lab-on-a-chip" device that could identify *Campylobacter* species and perform AST (Ma et al., 2020).

Campylobacter and *Salmonella* are the leading bacterial causes of foodborne outbreaks. According to the World Health Organization official website, these two microbes are also the major causes of human diarrheal diseases in the world (World Health Organization, 2018, 2020). Poultry is one of the most common reservoirs of these two pathogenic bacteria due to the higher body temperature of the hosts (Skirrow, 1977). According to a study about meat consumption in

the past decade (2010-2020), chicken consumption had the highest increase, especially in North America (Basu, 2015; Milford et al., 2019). Since chicken is the common food source for both *Campylobacter* and *Salmonella* as well as the commonly consumed meat type worldwide, it was selected as the target food product for investigating *Campylobacter* and *Salmonella*.

1.2 Research Hypotheses and Objectives

There are three main hypotheses in this thesis project. (1) The chromogenic-agar based microfluidic device can function normally with successful injection using an external pump and backflow-prevention zig-zag channels. (2) Identification and AST of *Campylobacter* and *Salmonella* by using pure bacterial mixture can be performed successfully in the microfluidic chip. (3) The on-chip AST performed by using spiked poultry samples can provide consistent testing results to that by the reference methods.

To address these hypotheses, three objectives needed to be achieve: (1) selecting the most suitable materials to fabricate the chromogenic agar-based microfluidic device; (2) testing the performance of the microfluidic device on identification and AST using pure bacteria culture; and (3) using spiked food samples to perform AST to evaluate the categorical agreement rate between the conventional MIC tests and on-chip AST.

CHAPTER 2. LITERATURE REVIEW

2.1*Campylobacter* and *Salmonella*

2.1.1 Characteristics of *Campylobacter* and *Salmonella*

*Campylobacter*s are Gram-negative bacteria and belong to the family *Campylobacter*aceae (Kaakoush et al., 2015). *Campylobacter* was firstly described by Theodore Escherich in 1880. The word '*Campylobacter*' is derived from the word 'kampylos' in Greek, which means 'curved' (Olson et al., 2008). It consists of 26 species, 2 provisional species, and 9 subspecies, in which *Campylobacter jejuni* is the major pathogenic species of *Campylobacter* that induces diseases in humans. *Campylobacter* species generally have spiral, rod-shaped, or curved shapes with flagella (or flagellum) including single polar flagellum, bipolar flagella, or no flagellum. The type of flagella (or flagellum) varies among different species. *Campylobacter* species do not form spore and have a size of approximately 0.2 to 0.8 μm width by 0.5 to 5 μm length (Kaakoush et al., 2015). *Campylobacter* is a microaerobic bacterium (Guccione et al., 2008). There is a very specific requirement towards the atmosphere condition for its growth. The carbon dioxide level is from 3-10%, oxygen level ranges from 3 to 5%, and nitrogen concentration is from 80 to 85%. The optimal growth temperature for this microbe is 37-42℃ (Garénaux et al., 2008). *Campylobacter* is fastidious and needs ~18 hour to enter the stationary phase under the optimal growth condition (Fitzgerald, 2015). *Campylobacter* metabolizes via a respiratory and chemoorganotrophic way and acquires energy from amino acids or tricarboxylic acid cycle intermediates, but not from carbohydrates (Nachamkin et al., 2008). *Campylobacter* also needs to be supplemented by hydrogen, formate, or succinate as electron source. The commonly used culture media to optimize *Campylobacter* growth in the laboratory include Müller Hinton (MH) broth, Brucella broth, and Brain-Heart Infusion broth (Sahin et al., 2017).

Campylobacter spp. are commensal organisms that can be routinely identified in the intestinal tract of different animals, including avian species, sheep, swine, and cattle. These are the most common hosts for *Campylobacter* spp. probably due to their relatively higher body temperature than others (Silva et al., 2011). Therefore, these animal species are the potential sources for water, food, and environmental contaminations. *Campylobacter* can still survive through some physiological progresses such as entering the viable-but-non-culturable (VBNC) state (Jackson et al., 2009) or forming biofilms (Reuter et al., 2010).

Salmonella is a non-spore-forming, facultative anaerobe that belongs to the family Enterobacteriaceae. In 1880, it was firstly observed by Karl Joseph Eberth from specimens of patients with typhoid fever (Daniel F. M. Monte & Fábio P. Sellera, n.d.). Salmon and Smith cultured *Salmonella* successfully from pigs which had died of hog cholera in 1885 (Merchant, 1950). Shortly afterwards, in 1892, S. Typhimurium was isolated by Loeffler from an infected mouse (Burrows, 1963). In the honor of American veterinary bacteriologist Daniel E. Salmon, the genus name '*Salmonella*' was proposed by Lignieres in 1900 and become formal in 1934 (Swayne, 2013).

Salmonella has a rod shape and a range in diameter from around 0.7 to 1.5 μm, with a length of 2 to 5 μm (Fàbrega & Vila, 2013). There are around 2600 serotypes that have been identified with the use of the standard Kauffman–White scheme in the genus *Salmonella* (Barlow & Hall, 2002). Most of these serotypes have high adaptability to tolerate various conditions in animal hosts including humans (Allerberger et al., 2003; Key et al., 2020). The optimum temperature to support *Salmonella* growth ranges from 35-40 ℃ with a pH range from 6.5-7.5 (Juneja & Sofos, 2009). However, they can adapt to some extreme environmental conditions (Humphrey, 2004). Most *Salmonella* serotypes are observed generally to grow over temperature ranging from 5-47℃ (Quinn, 1994), and the growth of some serotypes can be found even at 4℃ (Chen et al., 2013). It also has a board pH range for growth, which is from 3.8-9.5 (International Commission on Microbiological Specifications for Foods, 1996). In saline condition, some *Salmonella* could tolerate the NaCl level around 8% (Tortora et al., 2018). *Salmonella* has the ability to produce hydrogen sulphide, metabolize citrate as its sole carbon source and lysine as a nitrogen source (Ewing, 1986). For optimal growth of *Salmonella*, widely-applied culture media such as tryptic soy broth (TSB) and Luria Bertani (LB) broth can be used in the laboratory (Gurtler, 2009).

Salmonella are predominant in different food sources, including poultry, eggs and dairy products (Antunes et al., 2003; Holschbach & Peek, 2018). Other food sources such as fresh fruits and vegetables can also be contaminated by the transmission of *Salmonella* (Pui et al., 2011). In general, animal food sources such as poultry, swine, and cattle are the prime sources of *Salmonella* infections. The pathogens can be mainly disseminated through uncooked animal products, trade in animals etc (Heredia & García, 2018). The abattoirs are considered as an important sources of

Salmonella contamination due to frequent slaughtering process, massive organs and carcass, and transfer of live animals (McEvoy et al., 2003).

2.1.2 Adverse health effects and public health burdens of *Campylobacter* and *Salmonella* infections

Campylobacter is a commonly reported gastrointestinal bacterial pathogen worldwide. Campylobacteriosis is mainly caused by thermo-tolerant species *C. jejuni* (95%), followed by *C. coli* (4 %) and *C. lari* (<1%).(J. E. Moore et al., 2005)

Infections can occur with a dose as low as 800 CFU, or even 360 CFU that was observed in some volunteers with symptoms of diarrhea (Black et al., 1988). The clinical symptoms of campylobacteriosis that are commonly found in humans include abdominal pain, acute watery or bloody diarrhea, and fever. These symptoms occur within 2 to 5 days after the ingestion of food or water contaminated with *C. jejuni* (Robinson, 1981).

In general, the symptoms are self-limiting and can be resolved within 3 to 10 days. Antibiotics are not required in most of the cases. If there is a need for antibiotic, erythromycin and fluoroquinolones are often applied. Besides gastroenteritis, *Campylobacter* infections may lead to some other adverse health implications such as Guillain-Barré syndrome (GBS) (Nachamkin et al., 1998), Miller Fisher syndrome (MFS) (Godschalk et al., 2007), inflammatory bowel diseases (Kaakoush et al., 2014), and colorectal cancer (He et al., 2019). GBS is an acute disease with symptoms of fever, pain, and weakness that may develop into paralysis, while MFS is characterized with ataxia, areflexia, and ophthalmoplegia (Berlit & Rakicky, 1992). These two syndromes may happen with an approximate rate of 0.1% after 2-3 weeks' infections (Keener et al., 2004).

Campylobacter is one of the major foodborne pathogens in North and Central America (Kaakoush et al., 2015). Based on a 10-year investigation of pathogens in USA (1998 to 2008), the annual number of campylobacteriosis was estimated to be 8,463 hospitalizations and 76 deaths among 845,024 infection cases (Batz et al., 2012). From 1996 to 2012, the U.S. Food-Borne Diseases Active Surveillance Network claimed that the annual incidence rate for *Campylobacter* infections was 14.3 per 100,000 population (Crim et al., 2014). The estimated annual cost induced

by campylobacteriosis was about $1.7 billion in the United States (Hoffmann et al., 2012). Further, the incidence of campylobacteriosis had a 14% increase in 2021 compared to that from 2006 to 2008. In contrast, the incidences of other pathogens such as *Cryptosporidium*, *Listeria*, *Salmonella*, *Shigella*, Shiga-toxigenic *Escherichia coli* (STEC) O157:H7, and *Yersinia* infections had a decreasing trend at the same time (Crim et al., 2014).

Salmonella has two major species, namely *Salmonella enterica* and *Salmonella bongori*. *Salmonella bongori* is mainly found in cold-blooded animals and do not commonly associate with human infections (Chan et al., 2003). *S. enterica* contain 6 subspecies and these subspecies are responsible for more than 99% of human salmonellosis (Lamas et al., 2018). *S.* Typhimurium and *S.* Enteritidis are two representative serotypes (Heredia & García, 2018). *S.* Typhi and *S.* Paratyphi are characterized as typhoid *Salmonella* and humans are the only reservoir. The rest of *Salmonella* serovars are non-typhoid types and animals are the reservoir (Eng et al., 2015).

Two major syndromes caused by *Salmonella* infections in humans are typhoid fever and diarrheal disease. Typhoid is caused by ingestion of *S.* Typhi bacteria. The common sources would be contaminated water, food products made of animals, or close contact with the infected carrier.

The typhoid disease manifests 1 to 2 weeks after the infections in humans (Parry et al., 2002). The clinical implications include malaise and abdominal pain. Some other symptoms that may or may not occur along with the previous symptoms include headache, myalgias, nausea, anorexia, and constipation. Diarrhea is the occasional case and usually takes place after the infections in the immunocompromised population. In most cases, fever is mild at the beginning of the infection and worsening as salmonellosis progresses (Coburn et al., 2007). Diseases may resolve within varied periods, but carriage of the bacteria can possibly continue for months or years. For treating *S.* Typhi infection, the primary option of antibiotics is fluoroquinolones (Piddock, 2002). Nalidixic acid and other antimicrobial agents are also used (Kadhiravan et al., 2005).

Non-typhoidal diseases induced by *S. enterica* facilitates colonization in intestines (McGovern & Slavutin, 1979). Infected patients have symptoms between 6 and 72 h after consumption. Symptoms are characterized by crampy, abdominal pain, diarrhea, or even bloody diarrhea, nausea, and vomiting. Inflammations can also take place in ileum and large bowel (Boyd, 1985). These symptoms usually last about 5 to 7 days and patients recover spontaneously.

Common treatments involve oral or intravenous rehydration to prevent fluid loss due to electrolyte imbalances. Specific antimicrobial treatment is needed only in the case of the existence of invasive diseases (Coburn et al., 2007).

In a global scale, 155,000 deaths occurred in 93.8 million cases of gastroenteritis caused by *Salmonella* every year, and 80.3 million cases were foodborne (Majowicz et al., 2010). To be specific, *Salmonella* has an estimation of causing 627,200 cases of infection and cost $846.2 million CAD annually in Canada. This amount even exceeds twice of the estimated cost for all other foodborne illnesses combined (Martin et al., 2004).

2.1.3 Antimicrobial resistance in *Campylobacter* and *Salmonella*

In addition to the high infection rate, *Campylobacter* bacteria also pose a challenging threat to the public health because of the continuous development of antimicrobial resistance. Numerous studies have found significantly high level of *Campylobacter* with antimicrobial resistance in food isolates.

In a study of AMR *Campylobacter* from local broiler chickens and relevant products in different regions in Canada, a total of 9615 samples were collected and 1460 samples among the total were tested for antimicrobial susceptibility. Tetracyclines were the most common antibiotics that *Campylobacter* could resist (45.8% - 48.7%) (Dramé et al., 2020). In a report by USDA-NARMS (National Antimicrobial Resistance Monitoring System), 20–27% *Campylobacter* isolates from chickens were resistant to quinolones, 42–45% were resistant to tetracyclines, and 1.3% were multidrug-resistant (Doyle, 2014). In another study, AMR genes of 32,256 *C. jejuni* and 8,776 *C. coli* have been identified. A total of 68% *C. coli* and 53% *C. jejuni* isolates contained AMR markers while 15% *C. coli* and 2% *C. jejuni* had multi-drug resistance (van Vliet et al., 2022).

Antimicrobial resistance in *Salmonella* can be traced back to an early time, and has constantly been a serious issue for public health. In a study for investigating *Salmonella* outbreak in the USA from 1984 to 2002, 10 out of 32 were *Salmonella*-resistant outbreaks with a hospitalization rate of 22% of 13,286 persons. The rest 22 outbreaks caused by pansusceptible *Salmonella* strains made 8% of 2,194 persons hospitalized (Varma et al., 2005). Further, there

were estimated 4.95 million deaths in 2019 globally that were associated with antimicrobial-resistant bacterial infections. Within all the cases, 1.27 million deaths were directly attributable to AMR (Murray et al., 2022).

According to the *Salmonella* report from USDA-NARMS in 2014, 21.5% (1,077/5,001) of fecal samples had *Salmonella* isolates. In addition, 13% of these samples were multidrug resistant, and 66% of *Salmonella* isolates were not resistant to any antimicrobial tested. Among all the antimicrobial resistant samples, 28.8% of them were resistant to tetracycline while 17.9% were resistant to streptomycin. These two antibiotics had the highest percentage of resistance (Safety & Service, 2014).

Recently, outbreaks of fluoroquinolone-resistant *Salmonella* infections were reported in various regions, including the USA, Europe, and Asia (Casin et al., 1999; S. Chen et al., 2007; Hur et al., 2012; Varma et al., 2005). Due to the wide application of fluoroquinolones towards severe salmonellosis, emerging resistance to this antimicrobial raises concerns.

2.1.4 Transmission of antimicrobial resistance along the food and poultry supply chain

2.1.4.1 Contaminations of food from the environment

Food contaminations can come directly from antimicrobial-resistant bacteria in the environment. These antimicrobial-resistant bacteria exist in soil, water, and fecal materials from both humans and animals. Plant products may be contaminated as a result of irrigation water containing fecal materials (Quiroz-Santiago et al., 2009). Raw meat products may contain antimicrobial-resistant bacteria from fecal contaminations during slaughtering stage (HAFE et al., 1997). Antimicrobial-resistant bacteria can also be transferred to the food during the handing by the consumers. The origins may from other places or other foods defined as cross-contaminations (Verraes et al., 2013).

2.1.4.2 Contaminations due to food processing and preservation

Processing and preservations are techniques that aim to modify the food to a desirable state and extend its shelf life. Some bacteria can still possibly survive after processing and preservations

without inhibition of growth while other might be inactivated or stressed. Cell walls may be damaged in dead bacterial cells, and this leads to the liberation of antimicrobial-resistant DNA into the environment (Cadena et al., 2019).

The expansion of the concept of acquiring maximum nutrients and retaining tastes increases the demand for consuming raw or minimally processed foods. In these cases, raw food products such as fresh vegetables, fruits, and milk may be ingested without any thermal/non-thermal treatment. Thus, AMR bacteria may survive or their genes may remain and thus, increasing the risk for consumers to face resistance transfer (Verraes et al., 2013).

2.1.5 Different detection methods

2.1.5.1 Culture-based detection

Currently, culture-based detection is still the most widely applied method in clinical microbiology laboratories for detecting most pathogens from samples. Typically, agar-based media (e.g. nutrient agar) is applied for culturing a wide range of pathogens. Upon the completion of the growth, each colony will be investigated individually for identification. Differential media utilize biochemical indicator systems such as incorporation a specific nutrient (e.g. sugar) with a pH indicator (to sense the metabolite/digested nutrients/produced products) to indicate the likely existence of the target colonies more specifically. Culture media can also be selective. This could inhibit the growth of other microflora to maximize the likelihood of the isolation of the targeted bacteria (Váradi et al., 2017). Chromogenic agar offers rapid process for both isolation and detection. It surpasses the standard isolation media in the way of easy operation and no requirement for specially-trained personnel (Borman et al., 2021).

The reliability and range of application bases help culture-based methods constitute the majority of conventional testing for bacterial detection. They are still considered as the golden standard for bacterial identification. In contrast, this technique also has inevitable limitations such as considerable amount of operations, needs for labor, requirement for specific laboratory equipment and consumables, and long processing time. In addition, training is always necessary for the laboratory personnel to prepare and perform the tests or interpret the outcomes. Currently,

more rapid and user-friendly systems need to be developed regardless of the high application rate of culture-based methods (Rajapaksha et al., 2019).

2.1.5.2 PCR

PCR is another technique that has been widely applied in microbiological laboratories. It is a nucleic acid amplification technique for bacterial identification (Valones et al., 2009). The working principle is to isolate, amplify, and quantify a short section of the DNA sequence in bacterial cells. During the identification, the process is divided into three stages that are denaturation, primer annealing and primer extension. DNA will firstly be denatured by heating. After that, DNA will be extended by using primers and thermos-stable polymerization enzymes (Lee et al., 2011). In this sense, every double-stranded DNA is a target for the following cycles and amplification. Detection of the amplified DNA sequence will be performed via gel electrophoresis (Coleman & Tsongalis, 2006).

PCR is more rapid and specific than culture-based methods. The most apparent disadvantage would be the lack of differentiation between viable and non-viable cells since DNA presents in both dead and alive cells (Cangelosi & Meschke, 2014). Reverse transcription-quantitative PCR (RT-qPCR) can be applied to quantitatively measure viable bacteria cells by targeting mRNA (Adams et al., 2003). Compared with DNA, mRNA is more unstable and it has a short half-life measured in minutes (Belasco & Biggins, 1988). The existence of mRNA is thought to indicate the cell viability since mRNA degrades in dead bacterial cells. Therefore, the mRNA of various genes can be adopted as target genes for detecting VBNC bacteria. However, RT-qPCR also has limitations due to the difficulties in extraction and vulnerability of mRNA (Zhong et al., 2016). The matrix effect from the original samples can impact the efficiency of RT-qPCR (Yang et al., 2014). Another feasible assistance method that can solve this problem includes utilizing DNA amplification inhibitors in dead cells [i.e., double-stranded DNA intercalating dyes, propidium monoazide (PMA)]. PMA can penetrate the membrane of dead bacterial cells. With intensive light treatment, changes can be made in the structures of DNA by converting azido group to a nitrene radical. The nitrene radical then reacts with the DNA strand and forms covalent bonds between nitrogen and carbon. This makes DNA insoluble and further results in DNA loss in extraction and prevention of DNA polymerase to elongate the DNA (Askar et al., 2019). Based on

these factors, PMA-treated DNA in dead cells cannot be amplified via PCR procedure (Gao et al., 2021).

2.1.5.3 ELISA

ELISA stands for enzyme-linked immunosorbent assays. This assay is based on the specific interaction between antigens and antibodies to detect the target molecules (Tabatabaei et al., 2021). ELISA is easy for performance, provides faster outcomes and is relatively cheaper. The purity of antibody is important to a successful immunoassay. Additionally, the specificity of antibody is also vital to immunoassay. These can affect the specificity and sensitivity of the reaction that may give false positive results (Nielsen et al., 2004).

ELISA assays alone sometimes are not sensitive enough to detect trace analytes in the samples. Therefore, chemiluminescent and fluorescence-based immunoassays have been developed and applied to improve the sensitivity. Some other techniques such as the combination with nanomaterials are also used to further improve the detection limit. These nanomaterials provide more binding sites or higher selectivity to improve the overall signal intensity (Cinquanta et al., 2017).

2.2 Microfluidic system

Microfluidic devices/system is a growing field of research during the past 20 years, demonstrating a broad range of applications in agricultural, biological, chemical, medical, and environmental fields (Funes-Huacca et al., 2012; Hiltunen et al., 2018; Li et al., 2020; Mao et al., 2020; Salve et al., 2018). It is defined as a system that manipulates a small quantity of fluids on a micro- or nano- scale to control the chemical, biological and physical processes relevant to sensing (Song et al., 2018). Lab-on-chip (LOC) development was encouraged in this sense. LOC device is a network of micro-channels incorporated into the microfluidic chip. The scale is typically at sub-millimeter. One end of these channels links to the micro-environment located in the chip and the other end directs out through the chip. Fluids will be injected through these pathways and exit from the microfluidic chip. Inside the chip, fluid will be directed, mixed, and manipulated to create an

automated and high-throughput system that involves processes such as sample preparation, reagent mixing, detection, etc (Sanjay et al., 2018). Due to its miniaturized nature, LOC has a variety of remarkable features including portability, precise-controlled micro-environments, low-cost, cost-effective procedure, operation-friendly for non-skilled personnel, etc (Dou et al., 2014; Jin et al., 2020; Sanjay et al., 2015; Shen et al., 2014). These benefits provided by LOC facilitates numerous applications in different fields including human health tests, environmental analysis, disease diagnosis, as well as offer a feasible option for platform selection of point-of-care (POC) applications (Dou et al., 2014; Jalal et al., 2017; Marle & Greenway, 2005).

2.2.1 Materials

During the past two decades, numerous materials have been applied in microfluidic field (Sollier et al., 2011). At first, silicon and glass substrates were the initial options as the materials for developing microfluidic device. With the expansion of this field, other materials were introduced gradually. These materials can be categorized into three general types including inorganic materials, plastic polymer materials, and paper (woven fabrics). The inorganic material category can be divided into glass, silicon, and ceramics. Polymer-based materials include elastomers and thermoplastics. The paper-based microfluidic device is a developing area where paper can be combined with other materials to adopt different technologies (Ren et al., 2013). For the selection of the materials for a microfluidic system, we need to consider the functions needed, the way of material integration and the required applications. Aside from these basic factors, physical properties such as electrical conductivity, air permeability, flexibility, optical transparency, solvent compatibility, and cellular compatibility also need to be considered carefully based on the actual requirements and applications.

2.2.1.1 Inorganic materials

2.2.1.1.1 Silicon

Silicon was the first material used for microfluidics while in other research areas, inorganic materials have been put into use a long time ago (Terry et al., 1979). The application of inorganic

materials for micro-channels existed before the concept of 'microfluidics'. In the study by He and others, quartz substrates were used for micromachining in liquid chromatography columns instead of the conventional packed column approach (He et al., 1998). Another study conducted by Tjerkstra and others shows the concentric hemispherical microchannels made by electrochemical silicon etching (Tjerkstra et al., 1998). A method for single-side wet etching of Si and SiO2 substrates was developed by Brugger and others (Brugger et al., 1998). In general, gas chromatography and capillary electrophoresis, and flow reactors micromachined in metal are the areas where glass or quartz capillaries are applied at first (Reyes et al., 2002).

The fabrication techniques to create patterns and structures for the silicon-cased microfluidic systems include substrate methods such as wet etching and dry etching or additive methods (e.g. stereolithography, two-photon polymerization, extruded droplet/filament technologies) (Channon et al., 2016; Padash et al., 2020). In some cases, a constant maintaining of temperature is essential to the tests (Shen et al., 2005; Vigolo et al., 2010). The high thermal conductivity of silicon can contribute to maintaining a uniform temperature distribution (Benson et al., 1999; Pipper et al., 2007). It is also able to be made into thin layers or membranes. The reduced thickness allows thermal mass to be lowered and increases temperature ramp rates (Tiggelaar et al., 2005). The elastic modulus value of silicon is high (ranging from 130 to 188 GPa) so that it has some difficulties in making silicon into active components in the microfluidic system (Hopcroft et al., 2010). However, silicon still has the mechanical properties to be applied in the fabrication of precise-definition components such as micro-valve (Oh & Ahn, 2006) and micro-pumps (Laser & Santiago, 2004). It can be also used for complicated 3D structures. In the study by Franssila and others, silicon was applied for making a silicon-glass hybrid microfluidic nebulizer chip (Franssila et al., 2006). In another study, Tiggelaar and others developed a micro-reactor made of silicon for high-temperature catalytic partial oxidation gas phase reactions (Tiggelaar et al., 2005). Moreover, Zuta and others' designed platform that involved a silicon nitride micro-ring resonator (Zuta et al., 2010). In another study performed by Sainiemi and others, silicon was used to make electrospray ionization tips (Sainiemi et al., 2011).

Silicon surfaces can be modified to achieve desired purposes. In the study by Bimbo and others, porous silicon surfaces can be changed from hydrophobic to hydrophilic by using an easy stabilization method named thermal oxidation. In physiological situations, this modification favors

drug delivery (Bimbo et al., 2011). Wu and others found that carboxylic acid functionalities created by hydrosilylation to the porous silicon surface enabled anticancer drugs (e.g., doxorubicin and daunorubicin) to attach onto the surface covalently (Wu, Andrew, Buyanin, et al., 2011; Wu, Andrew, Cheng, et al., 2011). From the perspective of optical properties, visible light cannot transmit through the silicon, but infrared can. This makes the conventional fluorescence detection difficult for embedded structures (Cullis & Canham, 1991; Nge et al., 2013). This can be solved by integrating transparent parts like glass or polymer-based materials with silicon to create a hybrid device (Anderson et al., 2011).

2.2.1.1.2 Glass

Glass substrates have been preferably applied in the field of miniaturized analytical systems for electrophoresis in the recent years (Effenhauser et al., 1995; Harrison et al., 1993; Jacobson et al., 1998; Jacobson, Koutny, et al., 1994; Ramos-Payán et al., 2018). The common operations for the fabrication of glass-based microfluidic devices are usually subtractive methods (e.g., wet or dry etching) or additive methods (e.g., metal or chemical vapor deposition) for structure creation (Queste et al., 2010). Glass has the amorphous characteristics. The etched glass has smooth and rounded walls except for being treated with special techniques (Mu et al., 2009). The elastic modulus of glass is large and varies with its compositions. Thus, the hardness makes glass-based chips to require additional parts like pumps and valves (Grover et al., 2003, 2006). It is bio-compatible and is not permeable to gas. Qu and co-authors fabricated a type of glass chip using photolithography and thermal bonding that could separate red blood cells from whole blood with a high efficiency based on the native magnetic properties of blood cells (Qu et al., 2008). In the study by Li and others, a microfluidic chip of an ultraportable flow cytometer made by glass was established. The small surface roughness provided by glass significantly enhanced optical performance of the on-chip micro-lens' (Li et al., 2023). Wang and others developed a glass microfluidic chip that could be applied in electrophoresis and laser-induced fluorescence detection for separation and determination of β-casomorphins in cheese (Wang et al., 2011).

Glass can insulate electricity and is optically transparent. The insulation property of glass allows high-voltage applications that offer fast and effective separations (Effenhauser et al., 1993; Jacobson, Hergenroder, et al., 1994). On-chip electrophoretic separations adopt various modes that

include micellar electro-kinetic capillary chromatography (Moore et al., 1995). open channel electrochromatography (Jacobson et al., 1999), and capillary gel electrophoresis (Effenhauser et al., 1995). Optical transparency permits glass-based microfluidic chips to have sensitive optical detection (Chiem & Harrison, 1997). Based on these characteristics, one of the major functions of the glass-based microfluidic chip is capillary electrophoresis (CE). Conventional CE has the limitation that it can only analyze one sample or lane at a time. On-chip CE can solve this issue by increasing the throughput. Woolley and co-authors improved the limited sample capacity by establishing capillary array electrophoresis. They developed a series of parallel silica capillaries to carry out multiple separations simultaneously (Woolley & Mathies, 1994). Miniaturized size also made this method more cost-effective (Woolley et al., 1997). With the electroosmotic flow, on-chip CE provides valve-free injection. Glass also has good resistance to organic solvents and the ability to have metal depositing. Moreover, its high thermos-conductivity and stable electroosmotic mobility makes glass-based microchannel perform better than that made of other materials (Ren et al., 2013).

Glass and silicon share some similarities like the nature of hardness, which limits their applications in the microfluidic field. One of the major problems preventing their proliferation is the expensive fabrication process. Dangerous chemicals (e.g., hydrogen fluoride) are utilized from the beginning of the entire process so that the working environment requires high-standard protective facilities (Luitz et al., 2020; Yuen & Goral, 2012). Another aspect is the later bonding process that is usually more difficult for glass and silicon due to the need for high temperature, high pressure, and an extremely clean environment (Howlader et al., 2010; Serra et al., 2017). The same theory also applies to the fabrication of small components. Valves are hard to be made in the glass-based or silicon-based microfluidic device. The possible solution to improve these drawbacks can be integrated with other materials such as a glass-polymer chip. Further, glass and silicon are not gas-permeable which is not suitable for cell cultivation for a long time.

2.2.1.1.3 Ceramics

Ceramic-based microfluidic chips are usually made of low-temperature co-fired ceramic (LTCC). LTCC is a material based on aluminum oxide in the form of laminate sheets that can be modified, assembled, and fired together at an ascending temperature (Fakunle & Fritsch, 2010).

With the laminar property, LTCC can form a complex three-dimensional structure. In the ideal situations, the layer amount is unlimited and each layer can have individual quality-control inspection before being utilized. This helps build a more complicated integration that includes electronic circuits and three-dimensional fluid networks. In addition, this characteristic improves yielding and makes the fabrication of ceramic-based chips more cost-effective in the way that the previous-processing effect can be avoided. Moreover, LTCC does not need clean-room environment, toxic reagents, or corrosive chemicals that are usually required in photolithographic processes. Thus, ceramics surpass silicon and glass in these aspects (Gongora-Rubio et al., 2001). However, ceramic also suffers from opacity and thus has difficulty in optical applications (Patel et al., 2006). It might also shrink during the firing process (Correia et al., 2004). Henry and others constructed a capillary electrophoresis system made of ceramic that could achieve highly efficient separation for catechol and dopamine based on electrochemical reactions (Henry et al., 1999). Almeida and others developed an LTCC-based device based on the potentiometric procedure to analyze sulfamethoxazole and trimethoprim in aquaculture waters (Almeida et al., 2011).

2.2.1.2 Plastic Polymer

Polymers are long-chain materials that were introduced in chip fabrication several years later than silicon or glass-based microfluidic chip and have earned increasing attention in microfluidics in the past decade. There is a large variety of polymers that provide enough choices and flexibility in finding a suitable material with desired characteristics (Sollier et al., 2011). Polymers are also advantageous over glass and silicon in the way of a relatively low cost, ability to undergo massive production (e.g. injection molding, etc.), and compatibility with chemical modifications (Ren et al., 2013). Based on their physical properties, polymers can be categorized into three groups named elastomers, thermosets, and thermoplastics.

2.2.1.2.1 Elastomers

Elastomer is composed of cross-linked polymer chains. The intermolecular forces in between the chains are low. Thus, elastomers can have high-degree elongation or compression when external forces are exerted onto them and return to the original shape after the forces are

withdrawn (Özdemir, 2020). The most popular and widely-used elastomer in microfluidics is polydimethylsiloxane (PDMS) (McDonald & Whitesides, 2002).

A significant superiority of PDMS is its easy and cost-effective fabrication process (McDonald et al., 2000). PDMS-based microfluidic chip is reproducible with a pre-established mold. PDMS has a low elastic modulus (300−500 kPa), which makes the fabrication of small components such as valves and pumps easy (Araci & Quake, 2012). Its low surface tension also facilitates the peeling step in fabrication. The device mold is made in advance via photolithography methods. During the fabrication, PDMS is casted onto the mold and peeled off after heating at a mild temperature for a period of time. The overall cost of PDMS chip fabrication is lower than silicon or glass platform (Xia & Whitesides, 1998). PDMS slabs can be attached to another PDMS piece or sealed to glass and other substrates reversibly by contacting two objects and gently pressing them. PDMS slabs can also have irreversible bonding to another PDMS slab, glass, or silicon via plasma cleaning treatment (McDonald et al., 2000). Different PDMS slabs can be stacked to each other to achieve multilayer structures for creating a complex channel system (Wu et al., 2003).

PDMS has another special property which is its permeability of gas. This can benefit oxygen and carbon dioxide transfer and facilitate bacteria cultivation or cellular studies. However, this property is a double-edged sword that bubbles formed from gas transportation through PDMS can be a problem (Berthier et al., 2012). PDMS also has hydrophobic nature so that it is susceptible to nonspecific adsorption and can be penetrated by hydrophobic molecules (Roman et al., 2005). The resolution for addressing these issues is surface modification. There are three main types, namely, surface activation, physical modification, and chemical modification (Wong & Ho, 2009).

The biomedical analysis is one of the areas that PDMS is widely used. Various aims such as cell analysis or DNA extraction can be achieved. Perez-Toralla and others used PDMS-based chip for capturing cell-free DNA of serum samples from cancer patients and carried out subsequent PCR test to determine the existence of target genes (Perez-Toralla et al., 2019). Fiddes and others utilized PDMS to fabricate a device that contained micro-channels with circular cross-sections by soft lithography to grow endothelial cells on the internal part of micro-channels (Fiddes et al., 2010). Moore and others developed a microfluidic device using PDMS that provide a condition where immune cells and reagents could constantly pass by the held tumor fragments during the

confocal microscopy. The permeability of PDMS mimics the *vivo* environment (N. Moore et al., 2018). PDMS-based microfluidic device is also proliferated in food analysis. Sayad and others developed a microfluidic device with a centrifugal system integrated with loop mediated isothermal amplification (LAMP) for colorimetric detection of pathogenic bacteria. This device has a limit of detection (LOD) of 3×10^{-5} ng/μL DNA (Sayad et al., 2018). Asgari and others established a microfluidic chip based on surface-enhanced Raman spectroscopy to separate and detect the pathogenic bacteria (*Escherichia coli* O157:H7) in romaine lettuce with a LOD of 0.5 CFU/mL (Asgari et al., 2022). Novo and others designed a PDMS-based microfluidic chip using enzyme-linked immunosorbent assay and chemiluminescence reaction to detect mycotoxins ochratoxin A in wine and beer. Its limit of detection was determined to be 0.85 ng/mL (Novo et al., 2013).

2.2.1.2.2 Thermosets and thermoplastics

Thermosets such as SU-8 photoresist and polyimide have worked as negative photoresists before being applied in the microfluidic field. They are defined as network-forming polymers. When they are heated or radiated, the molecules with the thermosets can cross-link to form a rigid structure. This form is considered to be 'set'. Once the structure has been shaped, it cannot be dissolved and re-constructed again (Jackman et al., 2001). In general, thermosets can tolerate high temperature and maintain normal functions. Similar to PDMS, these materials are also optically transparent. Moreover, they are resistant to many solvents (Becker & Gärtner, 2008). They have high strength and thus are suitable for making structures with high-aspect ratio, but this property also makes them inappropriate to fabricate diaphragm valves. Additionally, their costs are relatively high so they are not often the prior options for microfluidic devices (Sato et al., 2006; Zheng et al., 2010).

Unlike thermosets, thermoplastics can be re-constructed by heating even after being cured. To shape thermoplastics, they are heated to glass transition temperature followed by cooling. The common types of thermoplastics include polycarbonate (PC), poly(methyl methacrylate) (PMMA), polystyrene (PS), polyethylene terephthalate (PET), and polyvinyl chloride (PVC) (Becker & Gärtner, 2008). Compared with elastomers, they are also transparent (Boone et al., 2002; Liu & Fan, 2011). The permeation of small molecule in thermoplastic-based devices is usually lower (Li

et al., 2022). Thermoplastics cannot contact other substrates the same way as PDMS. Instead, they need techniques like thermo-bonding and glue-assisted bonding to seal the channels. These bonding techniques usually require working conditions that are milder and more easily achievable than glass and silicon (Neils et al., 2004; Tsao & DeVoe, 2009).

2.2.1.3 Paper

Paper is a flexible, highly porous cellulose-based material that has recently emerged as a feasible substrate option for microfluidic device (Martinez et al., 2010). Due to the porous nature, paper is good at wicking liquid. This property makes paper-based microfluidic chip to be equipment independent. Specifically, liquid sample can be loaded and distributed to the desired places without an external injection equipment. To achieve this, paper can be modified that certain parts are hydrophobic while the rest is hydrophilic. The aqueous solution can be guided precisely through the pre-determined pathways under capillary effects (Martinez et al., 2007). The fabrication of paper-based microfluidic devices is relatively simple. The techniques include photolithography (Martinez et al., 2007), etching (Abe et al., 2008), wax printing (Lu et al., 2009), ink jet printing (Li et al., 2010), laser treatment etc (Chitnis et al., 2011). Among these methods, lithographic methods can achieve the production of high–resolution devices, but the drawback is the high cost. In comparison, printing methods have a relatively more cost effective procedure (Li et al., 2012). This material also show environment-friendly property which can be burned or naturally degraded after disposal. It is cheap, widey-available at all the regions, and biologically compatible. Paper also allows the microfluidic device to equip different functions such as filtering, storing, and separation. Its natural white color makes it suitable to act as a background to offer a contrast in colorimetric detection methods (Pelton, 2009). Lopez-Ruiz and others developed paper microfluidic device that contains seven sensing areas with immobilized reagents. These reagents provided color alterations selectively when sample solutions were loaded in the desired areas. The images were captured by a smartphone and processed using a customized algorithm (Lopez-Ruiz et al., 2014). Another study of colorimetric paper microfluidic device by Dungchai and others utilized multiple indicators for a single analyte to generate different indicator colors at different analyte concentration ranges. This approach was approved to be effective in quantifying glucose (0.5–20 mM), lactate (1–25 mM), and uric acid (0.1–7 mM) in clinically relevant ranges (Dungchai

et al., 2010). Paper is easy to have chemical modification to achieve various experimental purposes. In the study conducted by Gabriel and other, they used chitosan to modify the paper surface to enhance the performance of colorimetric reactions associated with enzymatic bioassays (Gabriel et al., 2016). In another study, Figueredo and others used Fe_3O_4 nanoparticles, multiwalled carbon nanotubes, and graphene oxide to make the surface biocompatible and catalytic, so that the problem of lack in homogeneity on color measurements could be solved (Figueredo et al., 2016). Yang and others developed a paper platform modified with carboxymethyl cellulose to carry out immunoassay for testing tuberculosis. Carboxymethyl cellulose enables the paper to immobilize biomolecules (Yang et al., 2018).

Despite the adaptability of paper in colorimetry and luminescence detection, paper also has some limitations. For color-related devices, the pigment distribution may have non-uniformity in the tested regions on the paper which is challenging for naked-eye observation. In addition, data obtained from detectors may need further processes for better interpretation (Bruzewicz et al., 2008). Moreover, paper as a background will cause background noise to some extent, and thus, colorimetric sensors can be influenced with a low sensitivity and selectivity (Yang et al., 2017; Yetisen et al., 2013). The fabric structures of paper can block certain internal signals and thus lead to unsatisfactory detection. The analyte may also interact with fiber and cause adsorption of analyte non-specifically. This situation gets worse in the case of polar or charged analyte. This can also cause dilution during the sample transportation (Noviana et al., 2021). Moreover, loss of sample due to evaporation, low resolutions of micro-structures, and weak mechanical properties are also the challenges (Sher et al., 2017).

2.2.2 PDMS-paper hybrid microfluidic device application

Each material has its unique properties and drawbacks. On this basis, the accomplished single-substrate system can only offer limited functions. This situation encourages studies in developing hybrid-material microfluidic chips. With hybrid system, one device can include multiple merits into its functions while avoid potential limitations from one single material. PDMS-paper hybrid microfluidic chip is an emerging area.

Polymer or glass substrate itself needs to undergo complicated surface modification to have DNA primers or nanomaterials stably immobilized for the subsequent reactions. With the inclusion of paper, the wicking ability can effectively have those materials embedded. For example, nanosensors or primers can be immobilized in paper and integrated on the polymer-based microfluidic chip. The wicking effect also help uniform distribution of these products within the system (Zhan et al., 2017). In a previous study by Dou and others', the authors established a PDMS/paper-hybrid microfluidic chip with loop-mediated isothermal amplification (LAMP) for analyzing multiple bacteria (i.e., *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* type b) simultaneously. PDMS was utilized as the main body for reaction zones where LAMP reaction and detection took place. Paper was cut into small pieces and placed into the reaction reservoirs. It functioned as a porous 3D network to hold the DNA primers in for the later LAMP reaction. LAMP reaction resulted in a complex composed by pyrophosphate ion and manganese ions. Without free manganese ions, calcein could not be quenched and produced fluorescence. The visualized results could be observed directly or recorded by smartphone camera with UV light source. The authors also found that the presence of paper in this system could effectively increase the shelf life of the microfluidic chip compared with other chips they tested without paper (Liu et al., 2017).

The flexibility of polymers can allow the microfluidic chip to be fabricated in different forms to ease the test while the paper can make colorimetric results to be observed conveniently. Jolal and others designed a hybrid microfluidic device made of paper and polymer that could be disposed after each use. It applied colorimetric measurement to test urine and the corresponding results were analyzed by an Android app. Paper-based strips were commercial reagent test pads. They were placed in the microfluidic device for relevant tests. PDMS was used to make a micro-pump that could be controlled by finger-pressing to pull up the liquid sample. The urine sample can be loaded to the paper strip due to negative pressure with a constant small volume of 40 μL and glucose, protein, pH, and red blood cell could be determined with colorimetric reactions subsequently (Jalal et al., 2017).

Paper can also be used as a platform to contain nutrients for culture assays. With the combination of PDMS, this system surpasses the conventional agar petri dishes in the way of no need for aseptic environments and humidity, easy preparation, and portability. In the study by

Funes-Huacca and others, a microfluidic device was developed for bacterial growth and bacteriophage amplification. Samples from soil and shoes were collected by a swab as real-world environmental samples. PDMS and paper were the main body of the chip. PDMS can keep bacteria inside the growth zone, allowing gas to permeate freely. Paper was used to store nutritious media within the cellulose structure for bacteria growth. Different methods were carried out for estimating bacterial growth or phage growth including direct measurement via counting visible forming units, fluorescence measurement, and colorimetric detection (Funes-Huacca et al., 2012).

In another study, a microfluidic chip for drug screening based on cell culture assay was created by Hong and others. Conventional photolithography was used to fabricate the chip. Paper and PDMS were the main body of the chip for creating the injection port and culture arrays. HeLa cell was used and cultured in the microarrays. The anti-cancer drug doxorubicin was involved in performancing evaluation and its efficiency was revealed by the viability of HeLa cells after 8-hour exposure to the drug gradient. With microfluidic chips, reagent usage could be reduced and the drug gradient could be generated automatically. The cost of the conventional bioassays could be saved to a large extent (Hong et al., 2016). Gan's team established a hybrid microfluidic device made of filter paper, PDMS, and PMMA used for DNA extraction and subsequent PCR amplification. Fusion 5 filter paper was used for DNA extraction from different samples. PDMS was applied to reversibly bond the device as an adhesive agent in-between PMMA layers. This bonding design helped avoid any sample leakage during PCR. This chip could achieve DNA extraction efficiency of 8.1–21.8 ng of DNA yielded from 0.25–1 μL of human whole blood samples within 7 minutes. The yielding rate was higher than that obtained by the commercial kit (QIAamp DNA Micro kits). Other samples such as dried blood stains, buccal swabs, saliva, and cigarette butts could all be successfully processed (Gan et al., 2014).

2.2.3 PDMS-paper hybrid microfluidic device in agri-food industry

Recently, the applications of LOC in agri-food industry attract increasing attention for research purposes and practical uses. In agri-food industry, numerous products are associated with daily life including horticulture, crops, livestock, raw materials and food obtained from them etc. The safety, authenticity, and quality of these products often need careful inspections to meet the safety standard and market demand (Neethirajan et al., 2011).

2.2.3.1 Adulterant and authentication

Salve and others developed a paper-based microfluidic device printed using PDMS. Paper provided space for chemical reagents to be stored while PDMS was used to create hydrophobic barriers on the paper to form a test spot for the later colorimetric reactions. Adulterants in milk such as urea, starch, salt and detergent with a concentration ranging from 1 to 100 mg in 10 ml of milk sample were tested with the assistance of handheld colorimeter. The limit of detection of colorimetri for adulterants was 5 mg for urea, 17 mg for starch, 29 mg for salt and 20 mg for detergent in 10 mL of milk (Salve et al., 2018). Xie and others developed a PDMS-paper microfluidic device for detecting melamine in milk. PDMS was applied to form patterns onto the chromatographic paper. The patterns contained the detection zone, control zone, and waste zone. The role of paper was to hold the sample fluidic and provided space for the reaction. The entire detection process started with loading the milk sample mixed with different levels of melamine, followed by adding prepared citrate-stabilized AuNPs solution. After 1-minute reaction, a smartphone installed with a home-made app was used to record the images and analyzed them via colorimetric assays quantitatively within a LED-home-made box. The detection limit could achieve 0.1 ppm melamine in untreated aqueous solution and in liquid milk (Xie et al., 2019).

Another study conducted by Hu and others aimed to detect the addition of cheaper juices in pomegranate juice using a hybrid microfluidic device based on loop-mediated isothermal amplification (LAMP). PDMS provided hydrophobicity to create a proper pathway to direct liquid sample. Paper was folded into layer-structure to extract DNA. The results from LAMP were simplified by utilizing a Mn^{2+} and calcein-based fluorometric detection. The detection limit was 2 μL for fresh pomegranate juice and 5 μL for fresh apple and grape juice. The overall analysis required about 1 h. The cost for each test could be controlled around $4 USD and the reusable homemade portable heating device was around $15 USD (Hu & Lu, 2020).

Li and others developed a paper-based microfluidic device for detecting the concentration of glucose in fruits as an indicator of fruit quality. PDMS was firstly coated around a glass slide, which was pre-modified with allyltrimethoxysilane. PDMS provided the hydrophobic materials for cutting patterns. Paper was also patterned and loaded onto the PDMS base for later detection. Various reagents and samples were added to the paper and reactions took place in the paper part. The PDMS substrate was re-usable and paper substrate could be replaced for each test. The

generated color was recorded by a camera in a light-tight box for quantitative measurement. This method had a limit of detection of 3.12 mM (Li et al., 2017).

2.2.3.2 Microorganism

In a study performed by Zuo and others, a PDMS-paper hybrid microfluidic system was combined with aptamer-functionalized graphene oxide nano-biosensor for detecting multiple pathogens simultaneously. Two foodborne pathogens, namly *S. aureus* and *S. enterica* were chosen. PDMS provided a base to carve 32 micro-channels, inlet reservoirs and one shared waste reservoir. The chromatography paper substrate was pinched into small pieces and inserted inside each micro-well to facilitate the integration of nano-biosensors on the chip. The use of paper is a feasible alternative method compared with using surface treatment and immobilization of aptamer probe in a microfluidic system that is only made of PDMS or glass. The porous nature and high surface-to-volume ratio of paper could also enhance the reaction kinetics for a more rapid process. This device had a detection limit of 11 CFU/mL (Zuo et al., 2013).

Pang and others developed a self-priming PDMS-paper hybrid microfluidic chip that utilized mixed-dye-loaded loop-mediated isothermal amplification (LAMP) aiming at detection of multiple foodborne pathogens. PDMS acted as a waterproof material to make reaction chambers for sealing the liquid solutions inside the chip. The fabric structure of paper provided a suitable place for pre-loading and storing the primers. The emission spectral analysis and images were obtained under 455-nm blue light. Average fluorescence intensity in each circular area was selected as the results. This on-chip detection method had a limit of detection of 1000 CFU/mL. For real food sample application, this study utilized shrimps as the food matrix and spiked with *Staphylococcus aureus* and *Vibrio parahaemolyticus*. Different spiked concentrations were applied and the highest safety limit value of these two microbes was $10^3 g^{-1}(mL^{-1})$ (Pang et al., 2018).

In another study, Ma and others designed a culture-based hybrid microfluidic chip for carrying out identification and antimicrobial susceptibility testing (AST) of foodborne pathogen *Campylobacter*. PDMS was the main body of the chip that was used to have channels and cultivation chambers carved into and offered a gas-permeable environment for bacterial growth.

Paper was punched into small disks to be loaded with antibiotics and placed in each chamber below the agar for AST. *Campylobacter* was cultivated on a chromogenic agar loaded in the cultivation chambers. The agar has selectivity and can only change color from yellow to red when *Campylobacter* species exist. In this study, chicken was selected as the food matrix. With this real food sample, this device showed a detection limit of 10^4 CFU/25 g chicken sample with positive results after microaerobic incubation in 60 hours. In AST, it showed that the loaded antibiotics on the paper substrate could diffuse from the paper to the agar to perform a proper AST. The categorical agreement rates of all the selected antibiotics were all above 90% (Ma et al., 2020).

2.2.3.3 Allergen

Weng and others developed a paper-based microfluidic aptasensor for detection of food allergens and food toxins. For device fabrication and functions, the aptamers were bound to the aptamer-functionalized quantum dots (QDs) and mixed with the graphene oxide (GO). The fluorescence was quenched due to the Förster resonance energy transfer. The quenched fluorescence would be recovered when encountering the target protein in the food sample. The intensity depends on the concentration of the protein. The application of porous paper in the microfluidic device provided support for aptamer bound QDs-GO probes that avoided the use of surface modification, resulting in a more cost-effective procedure. PDMS acts as a hydrophobic barrier to keep the aqueous sample inside each well. The transparency of PDMS also facilitate fluorescence to pass. Fresh egg white, mussel tissue, and sausage were involved as the food sample to detect two allergens (egg white lysozyme, β-conglutin lupine) and two toxins (okadaic acid, brevetoxins). Only 10 μL of sample was needed and the test could be completed within 5 minutes (Weng & Neethirajan, 2018).

In the study conducted by Jiang and others, the peanut allergen Ara h1 was the target and detected by using an origami microfluidic electrochemical nano-aptasensor. This microfluidic device was composed by continuous folding of chromatography paper substrate. The paper acted as a base to have screen-printed electrodes attached onto. PDMS was used to generate a hydrophobic barrier in the paper substrate to protect electric connectors by preventing direct contact from solutions. In the measurement, cyclic voltammetry was used for qualitative analysis

while differential pulse voltammetry (DPV) was used for quantitative measurement. Once Ara h1 antigen binds to aptamers, the complex they formed lead to a change in the electrochemical signal. Ara h1 concentration directly related to the change in DPV. Cookie dough sample was selected for the spiking test. The corresponding results showed a good consistency, recoveries of the proposed aptasensor above 98%, and a relative standard deviations which was less than 5% (Jiang et al., 2021).

2.2.3.4 Toxin

In the study by Tang and others, the authors targeted on the aflatoxin B_1 (Tang et al., 2022) and pointed out that although conventional methods like chromatography, fluorescence quantum, and surface-enhanced Raman spectroscopy (SERS) technology were sensitive and could provide good repeatability, some drawbacks such as quantum dot yielding, poor SERS signals caused by the locations of aptamer and substrate, and expensive external equipment could not be avoided. In such case, they developed an immunomagnetic-bead-technology-based paper microfluidic device with the assistance of a smartphone to record colorimetric process associated with the starch-iodine reaction. Aflatoxin B_1 and the immunomagnetic beads had competitive relationship towards the capture of aflatoxin B_1 monoclonal antibody, followed by the attachment to a secondary antibody which is Glucose oxidase (GOx)-conjugated goat antimouse IgG. With the presence of oxygen, hydrogen peroxide was formed and used by starch-potassium iodide. Based on this reaction, a blue-violet color was produced and recorded by a smartphone. The advantage of this method includes quick response which takes about 6 minutes for color development, visible results which can be detected by naked eyes, and being able to have quantification test via analysis of the color change recorded by smartphone. The limit of detection (LOD) and quantification (LOQ) of this device were 9.45 and 12 ng/mL, respectively, which was similar to results reported in other studies using more established standard methods [e.g. LOD: 2.53 to 3.20 ng/mL (Deng et al., 2017), LOD & LOQ: 0.1 to 10 ng/mL (Babu & Muriana, 2011), LOD: 0.24 ng/mL (Wang et al., 2014), LOD: 0.00579 ng/mL (Li et al., 2018)]. In this sense, microfluidic devices can offer similar detection capability with a short reaction time.

In the study by Liu and others, they developed an integrated platform for detection of sulfur dioxide (SO_2). A microfluidic chip and battery-operated detection system were combined for the

entire process. This chip was based on paper, PMMA, and PDMS. The filter paper acted as a substrate for acid-base indicator to be coated onto and placed into a PMMA microchip. PMMA is rigid and acts as a strong reservoir for SO_2 to be injected into. PDMS was used to make a plug to remain the paper-holder in the position and keep sample inside. For the detection, injected SO_2 prompt a reaction with acid-base indicator that induced color change. The chip was then placed into detection system for image recording. These images were later transferred to a cell phone installed with a homemade RGB color analysis software measuring SO_2 concentration. In this study, results from 10 control samples showed a correlation coefficient of $R^2 = 0.9971$ within lower concentration (20 - 600 ppm) and $R^2 = 0.9920$ in the high-concentration range (600 - 5000 ppm). In the test involving real food samples, fifteen commercial samples were used for testing the feasibility of the developed platform. The obtained results showed an accuracy ranging from 95.71% to 99.64% (Liu et al., 2017).

Li and others designed a fluorescent-based hybrid microfluidic device made of PDMS and paper. PDMS was used to provide a hydrophobic base with groove patterns which were the detection regions. The paper substrate was used to immobilize the amine-capped carbon dots covalently and placed in the groove during the test. The device could receive fluorescence quenching response in the range from 1 to 300 μmol/L with the detection limit of 0.28 μmol/L. Folic acid was the target analyte and orange juice was chosen to be the food sample. In real food sample, this assay showed good reproducibility and stability (Li et al., 2020).

Overall, AMR of *Campylobacter* and *Salmonella* exerts significant burden to the public health, and therefore these two bacterial species were selected to be the target bacteria in this thesis project. Microfluidic chip test can be a feasible option to achieve rapid detection for avoiding AMR threat in an early stage. Combination of paper and PDMS as the material for the fabraciation of the microfluidic device can offset their disadvantages and have good compatibility. To allow the microfluidic device to carry out identification and antimicrobial test simultaneously, chromogenic agar-based microfluidic chip was developed and its performance was evaluated in this project.

CHAPTER 3. MATERIALS AND METHODS

3.1 Materials

The PDMS pre-polymer was purchased from Dow Sylgard (Dow Sylgard 184 silicone encapsulant clear kit, Canada) and was used for the fabrication of the microfluidic chips. Antibiotics, namely ampicillin sodium salt, ciprofloxacin, erythromycin, and tetracycline hydrochloride were purchased from Sigma-Aldrich (Sigma-Aldrich, Canada). These antibiotics were prepared as working solutions with desired concentrations, loaded onto paper disks, and placed into the incubation chambers. Chromogenic agar purchased from CHROMagar (CHROMagar *Campylobacter*; CHROMagar *Salmonella* Plus; CHROMagar, USA) was prepared and loaded on the antibiotic-added paper disks for subsequent tests. Raw, skinless, boneless chicken breasts were purchased from local grocery stores for tests using food samples. Sodium hypochlorite aqueous solution (10-15%) from Sigma-Aldrich (Sigma-Aldrich, USA) was diluted ten times to sterilize the chicken surface.

3.2 Preparation and characterization of microfluidic chip

3.2.1 Pattern design and manufacture of a microfluidic chip

Manufacture of microfluidic chip was conducted by three stages including chip structure design, silicon master manufacture and assembly of a hybrid microfluidic chip made of polydimethylsiloxane (PDMS) slabs and a glass slide.

The chip pattern was designed by using AutoCAD 2021 version (Autodesk, Inc., USA). There were three layers that composed the chip, namely, an injection layer, a chamber layer, and a glass layer. The injection layer and the chamber layer were made of PDMS while the glass layer was a conventional microscopic slide (75-mm length, 50-mm width, 1-mm thickness, Fisher brand, USA).

In the chamber layer, the patterns included an inlet port (Ø=1.5 mm) and an outlet port (Ø=1.5 mm), and 14 incubation chambers (Ø=4 mm). A main channel (Width=500 μm, Length=40mm) was developed to connect the inlet and outlet ports. Zigzag-shaped side channels (100 μm wide and 7 mm long) were designed to guide the injected fluids from the main channel

to 14 individual incubation chambers, and the excessive fluid was extruded from the air vent. The injection layer contained inlet and outlet ports (Ø=1.5 mm), 14 incubation chambers (Ø=4 mm), and 14 air vents (Ø=1.5 mm), with a 100-μm-wide channel to connect the incubation chambers and air vents. A photomask was used to have all the patterns made onto by CAD/Art Services, Inc. (Bandon, OR, USA). This photomask was used in the later master manufacture.

Standard photolithography was used to fabricate the silicon master. All the steps were conducted in a cleanroom (McGill Nanotools-MicroFab, Canada). For a start, about 5 mL of SU-8 2050 photoresist (Kayaku advanced materials. Inc, USA) was poured onto a clean silicon wafer (Ø=100 mm; University Wafer, USA). The SU-8 photoresist reagent was spin coated at 500 rpm for 5 s with an acceleration rate of 1305 rpm/s, followed by 2,000 rpm for 45s with an acceleration rate of 1305 rpm/s to achieve the pattern with thickness of 80 μm. After that, the photoresist reagent was soft baked at 65°C for 3 minutes and then baked at 95°C for 9 minutes. The wafer was cooled down to room temperature, followed by exposure to UV light (Dose, 215 mJ/cm^2) with the photomask containing designed chip patterns placed above. A post-exposure bake was conducted by heating the photoresist-coated wafer at 65°C for 2 minutes and then at 95°C for 7 minutes. Next, the photoresist-coated wafer was developed in SU-8 developer (Kayaku advanced materials. Inc, USA) to remove the unexposed photoresist. A hard bake was conducted to strengthen the attachment of the patterns on silicon wafer at 200°C for 5 minutes. With the obtained silicon masters, soft lithography was applied to fabricate injection layers and chamber layers by using PDMS. According to the manufacturer's protocol, the PDMS pre-polymer (Dow Sylgard 184 silicone encapsulant clear kit, Canada) was prepared in the proportion of 10:1 (wt/wt) of silicone elastomer and a curing agent. The mixture was blended thoroughly for at least 3 minutes. The pre-polymer was dispensed over the masters with aluminum foil wrapped around in advance for later steps. The injection layer required an amount of 18 g mixture while the chamber layer needed 30 g mixture. The masters with mixture were placed in a vacuum degassing chamber (ABLAZE 1.5 Gallon stainless steel vacuum degassing chamber; Ablaze, USA) to remove the gas inside pre-polymer. The degassed PDMS was cured on a hot plate (80°C) for 20 minutes to solidify the PDMS for pattern formation. The cured PDMS chip was then removed from the masters by gentle peeling. The incubation chambers, inlet port and outlet ports, and air vents were made by punching through the chip by Miltex biopsy punch (Ted Pella, Inc., USA) with defined diameters.

3.2.2 Assembly of the chip

Firstly, the two PDMS slabs were attached together by the treatment in a plasma cleaner (Harrick Plasma, USA) with 30-second suction and 30-second plasma treatment. PVDF membrane was punched into small disc (Ø=4 mm), autoclaved, and placed into each incubation chamber to support the loading of the chromogenic agar. To carry out on-chip AST test, a piece of pre-autoclaved paper disk (Ø=2 mm) was loaded with pre-determined antimicrobials, air-dried in a biosafety cabinet, and deposited on the top of the paper disk and PVDF membrane. After that, 20 µL of the chromogenic agar was loaded on the top. Lastly, the combined chip was sealed with a plain glass slide on the top using plasma treatment.

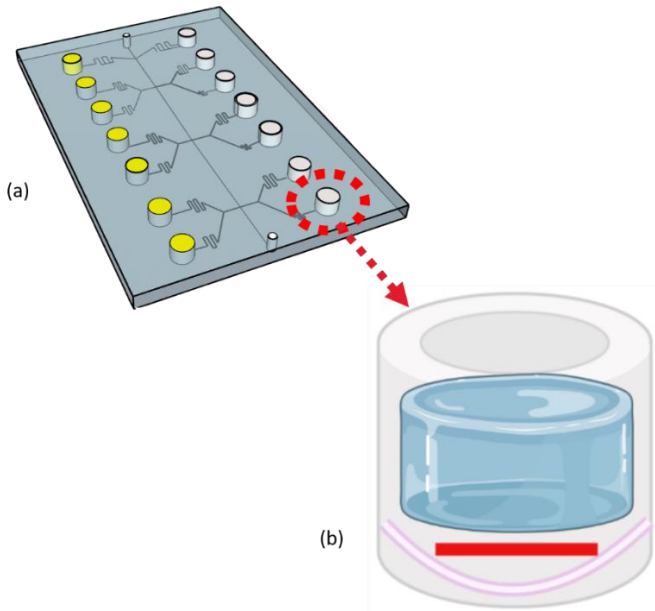


Figure 3.1 The intersection view of the assembled microfluidic chip. (a) yellow represents *Campylobacter* chromogenic agar while white indicates *Salmonella* chromogenic agar. (b) purple-white line indicates PVDF membrane. Red line indicates the paper disk with antimicrobials. Blue jelly-like part indicates the agar.

3.2.3 Injection process

The entire setting for the injection of bacterial culture consists of a syringe, a syringe pump, polyvinyl chloride (PVC) tubing from BD vacutainer without needle (Fisher Scientific, USA),

polypropylene elbow tubing (Nordson medical, USA), a petri dish (for collecting waste solution), a binder clip (serves as a stopper at the outlet port), and an assembled microfluidic chip. All the components were stored at sealed conditions when they were not under use and were sterilized under UV light for 30 minutes right before the usage. Polypropylene elbow tubings were immersed in 70% isopropanol for sterilization. The utilization of cut PVC tubing from BD vacutainer was to ensure that the internal part of the tubing was sterilized.

The polypropylene elbow tubing was inserted into the inlet and outlet ports of the microfluidic chip. It connected the inlet and outlet ports with the PVC tubing. One end of PVC tubing from the inlet port was connected to the syringe and PVC tubing from outlet port was placed into the waste container. The bacterial solution was contained in the syringe and constantly pumped using the syringe pump at a flow rate of 50 µL/min. The bacterial solution was firstly injected to full-filled the main channel. Then, the PVC tubing from outlet port was blocked by the binder clip. With the blockage, the bacteria solution flowed into each chamber. The injection was stopped when all the chambers were fully filled with the sample fluids. The inlet, outlet, and air vents were sealed with tape to prevent fluid leakage and liquid evaporation. After that, the microfluidic chip was incubated in a home-made CO_2 incubator (10% CO_2) at 42°C for 60 hours.

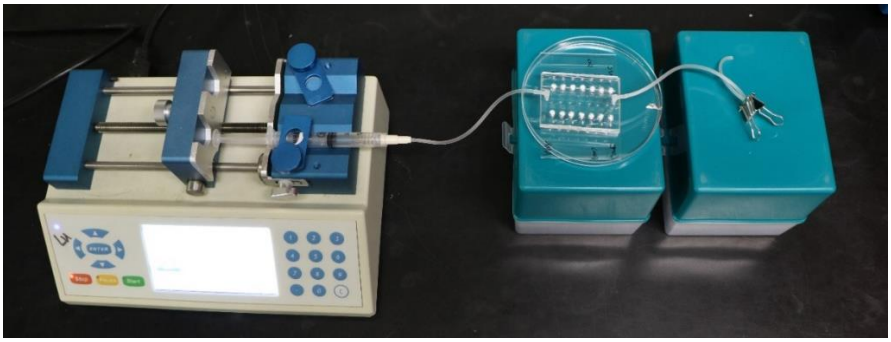
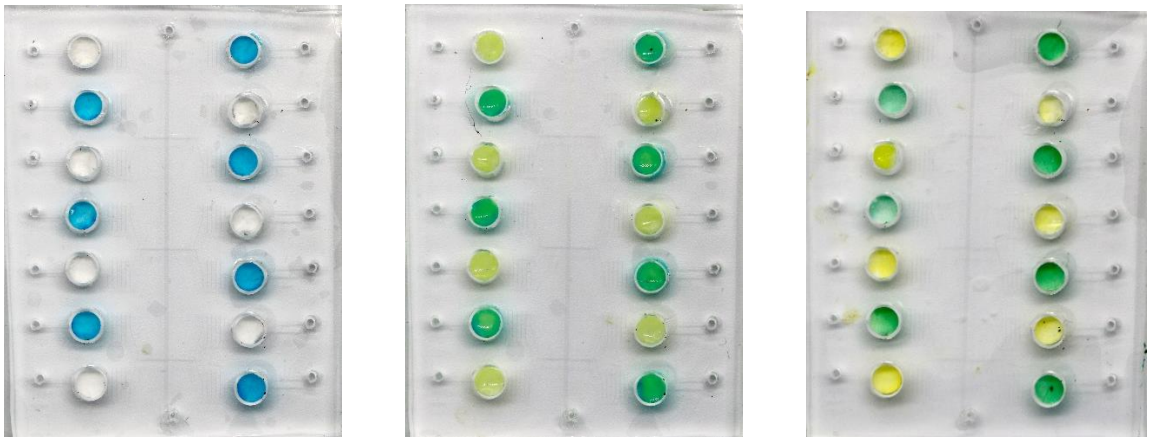


Figure 3.2 Demonstration of sample injection into the microfluidic chip. Image of the setup for sample injection consists of a syringe, a syringe pump, polyvinyl chloride (PVC) tubing, polypropylene elbow tubing (Nordson medical), a petri dish for collecting waste solution, and a binder clip served as a stopper at the outlet port.

3.2.4 Test of zigzag channel

Briefly, food dye was used to represent the antimicrobials. (**Figure 3.3**) Blue dye was added onto the paper discs and inserted into the separate incubation chambers. Yellow dye was injected into the entire chip. With the presence of blue dye and yellow dye, the paper disc loaded with blue dye turned into green while the other paper discs turn from white to yellow. The chip was then incubated 60 h.



(a) (b) (c)

Figure 3.3 Assessment of cross-contamination among incubation chambers. (a) Paper disks with or without blue dye were deposited into the incubation chambers of a microfluidic chip. The yellow dye solution was injected into the microfluidic chip until incubation chambers were fully filled. (b) The chambers loaded with blue dye were full of green dye due to mixing blue dye and yellow dye. The rest of the chambers were only filled with yellow dye. (c) The diffusion of green was investigated by keeping the microfluidic chip in a CO_2 incubator for 60 h, mimicking bacterial detection. The chamber with yellow dye did not change color, indicating that there was non-detectable green dye diffusing from the neighboring chambers. This validates that the design of microfluidic chips is free of contamination.

3.3 On-chip test with pure culture

3.3.1 Bacterial strains and cultivation conditions

A panel of *Campylobacter* strains were selected in this study, including *C. jejuni* (*n*=2) and *C. coli* (*n*=6). The strain names and isolation sources are listed in **Table 3.1**.

F For bacterial cultivation, Mueller-Hinton agar supplemented with 5% defibrinated sheep blood (MHBA) was applied. All *Campylobacter* strains were grown under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) at 37°C for 48 h. For the preparation of *Campylobacter* overnight culture, bacterial colonies were picked from MHBA plates and suspended in Mueller-Hinton broth (MHB), followed by incubation under microaerobic condition at 37°C for 16-18 h with constant shaking at 175 rpm.

Table 3.1 *Campylobacter* isolates used in this study.

Strain	**Source of isolation**	**Reference**
C. coli 171	pig	(Ma et al., 2020)
C. coli 314	pig	(Ma et al., 2020)
C. coli 1148	pig	(Ma et al., 2020)
C. coli 1330	cat	(Ma et al., 2020)
C. jejuni 1658	clinical	(Feng et al., 2018)
C. coli RM2228	chicken	(Miller et al., 2005)
C. jejuni F38011	clinical	(Feng et al., 2018)
C. coli RM1875	swine	(Mandrell et al., 2005)

A panel of *Salmonella* strains were selected in this study, including *S.* Enteritidis (*n*=2), *S.* Typhimurium (*n*=4), and *S.* Newport (*n*=1). The strain names and isolation sources are listed in **Table 3.2**.

For bacterial cultivation, Luria-Bertani (LB) agar was applied. All *Salmonella* strains were grown under aerobic condition (78% N_2, 0.04% CO_2, 21% O_2) at 37°C for 24 h. For the preparation of *Salmonella* overnight culture, bacterial colonies were picked from LB plates and suspended in LB broth, followed by incubation under aerobic condition at 37°C for 12 h with constant shaking at 175 rpm.

Table 3.2 *Salmonella* isolates used in this study.

Strain	**Source of isolation**	**Reference**

Salmonella. Enteritidis S173	poultry	(Oscar & Singh, 2009)
S. Typhimurium S806	poultry	This study
S. Newport S1299	poultry	This study
S. Typhimurium S1507	Bovine	This study
S. Typhimurium S1530	Human	This study
S. Typhimurium S1501	Bovine	This study
S. Enteritidis 43353	Bovine	(Draz & Lu, 2016)

3.3.2 Antibiotics

Ampicillin sodium salt, ciprofloxacin, erythromycin, and tetracycline hydrochloride were purchased from Sigma-Aldrich (Canada). Based on their solvent solubility, the stock solutions (640 µg/mL) of ampicillin, erythromycin and tetracycline were prepared in distilled water, while ciprofloxacin was dissolved in dimethyl sulfoxide.

The stock solutions were filtered through 0.22-µm sterile nylon syringe filters (Basix™, USA) for sterilization. Then, stock solutions were stored at -20°C until further use. For on-chip AST, the antibiotic solution was diluted by sterile distilled water to the desirable concentrations. Next, a sterile paper disk (Whatman no. 1 filter paper; ∅=2 mm) was loaded with 2 µL of prepared solution and air dried in a biosafety cabinet. Then, the prepared disk was inserted into the incubation chambers of the microfluidic chip. After that, either 20 µL of *Campylobacter* chromogenic agar medium (CHROMagar *Campylobacter*; CHROMagar, USA) or 20 µL of *Salmonella* chromogenic agar medium (CHROMagar *Salmonella* Plus; CHROMagar, USA) was individually loaded into each incubation chamber. The final concentration of antibiotics should be adjusted to 1×.

3.3.3 Specificity test

The on-chip identification test aims to target *Campylobacter* species and *Salmonella* species. *C. jejuni* F38011, *S.* Enteritidis 43353, *Staphylococcus aureus* MRSA-10, *Listeria monocytogens* ATCC 7644, *Escherichia coli* K12, and *Pseudomonas aeruginosa* PA14 were used to carry out the test. Existence of *Campylobacter* was shown by the chromogenic reaction that the

color altered from yellow to red. Existence of *Salmonella* was indicated by color alteration from white to mauve. To test the specificity of on-chip identification, various bacterial species were tested on both *Campylobacter* chromogenic agar and *Salmonella* chromogenic agar. Overnight culture of *S.* Enteritidis 43353, *S. aureus* MRSA-10, *L. monocytogens* ATCC 7644, *E. coli* K12, and *P. aeruginosa* PA14 were prepared in Luria-Bertani (LB) broth under aerobic condition at 37°C. Overnight culture of *C. jejuni* F38011 was prepared in Mueller-Hinton broth (MHB) under microaerobic condition at 37°C for 16-18 hours. The final concentration of overnight culture of each bacterial strain was adjusted to 10^8 CFU/mL. The overnight culture was then injected into the microfluidic chip and placed in a homemade incubator under the microaerobic environmental condition at 42°C for 60 hours. A Canon camera (model of EOS Rebel T6i) was used to record images of on-chip identification test at the beginning and the end of the incubation.

3.3.4 Optimizing supplement concentration for color observation

Chromogenic agar CHROMagar™ *Campylobacter* and CHROMagar™ *Salmonella* Plus were used to selectively cultivate and distinguish *Campylobacter* species and *Salmonella* species.

There are two major components in the chromogenic agar, namely nutrient base and supplements. Nutrient base can support bacterial growth and supplements provide selective agents and chromogens for selective bacterial differentiation. The selective agents such as antibiotics can help better identify the target bacteria by inhibiting the competing microbiota from complexed food samples. The chromogenic reactions between the target bacteria cells and chromogens lead to a color alteration from yellow to red (*Campylobacter*) or white to mauve (*Salmonella*). CHROMagar™ *Salmonella* Plus has another component, which is the white opaque supplement. It does not contain any selective agent and only provides a white background to facilitate the presence of mauve color. In order to enhance the color visibility of chromogenic reactions, optimization of supplement concentration (supplement that contains chromogens) was carried out. Six different concentrations were chosen to prepare the chromogenic agar. An aliquot of 20-µL chromogenic agar was added into the incubation chambers. A mixture of *C. jejuni* F38011 culture and *Salmonella* Enteritidis 43353 culture was injected into the microfluidic chip and incubated at 42°C for 60 hours. The final concentrations were adjusted to 10^8 CFU/mL and 10^5 CFU/mL for

Campylobacter and *Salmonella*, respectively. The mixed culture was used to mimic the actual scenario in food matrices.

3.3.5 Sensitivity test

To test the limit of detection (LOD) of this microfluidic chip, a range of concentrations of *C. jejuni* F38011 and *S.* Enteritidis 43353 ($10^2 – 10^8$ CFU/mL) were injected and incubated at 42°C for 60 hours under the microaerobic environment. Mixed culture was used in this test. For sensitivity test of *Campylobacter*, the background culture was *S.* Enteritidis 43353 (10^5 CFU/mL). For sensitivity test of *Salmonella*, the background culture was *C. jejuni* F38011 (10^8 CFU/mL). The color alteration of the on-chip identification assay was monitored with a time period of 12 hours for up to 60 hours. Images were obtained by using a Canon camera (EOS Rebel T6i) at each time point. The LOD was defined as the lowest initial concentration of *C. jejuni* F38011 and *Salmonella* Enteritidis 43353 that can generate distinguishable chromogenic reaction results.

3.3.6 Antimicrobial susceptibility testing (AST)

An on-chip AST was developed to determine the multidrug resistance (MDR) profiles of *Campylobacter* and *Salmonella* isolates. The procedure of the test was designed based on the CLSI protocols with some modifications (CLSI, 2018). In the MDR assay, three antibiotic classes were chosen to perform the test, namely ciprofloxacin, erythromycin and tetracycline for *Campylobacter* and ampicillin, ciprofloxacin and tetracycline for *Salmonella*. Two breakpoints namely "susceptible" and "resistant" determined by CLSI were chosen as the tested concentrations (McDermott et al., 2018; Tang et al., 2017). Antibiotic working solutions with pre-determined concentrations were prepared. A total of 2 µL of each working solution was pipetted onto a sterilized paper disc, followed by being air dried in biosafety cabinet and placed into the microfluidic chip. Next, 20 µL of chromogenic agar was loaded into each incubation chamber. The final concentrations of the antibiotics in the chip were the same as the CLSI breakpoints. A mixed culture with final concentration of 10^8 CFU/mL of *Campylobacter* and 10^5 CFU/mL of *Salmonella* were incubated in the microfluidic chips at 42°C under the microaerobic condition for 24 hours (CLSI, 2018).

The antibiotic breakpoints of two species are listed in **Table 3.3**. A total of 12 isolates of *Campylobacter* and *Salmonella* (**Table 3.1, 3.2**) were tested using an on-chip MDR assay. Conventional broth micro-dilution method was conducted in parallel to validate the on-chip MDR results. The MDR results were determined based on the presence of color.

Table 3.3 MIC breakpoints were obtained from Clinical Laboratory Standard Institute (CLSI) (PA, 2016, 2020).

Campylobacter		
	Susceptible breakpoint (μg/mL)	Resistant breakpoint (μg/mL)
ciprofloxacin	≤1	≥4
erythromycin	≤8	≥32
tetracycline	≤4	≥16
Salmonella		
	Susceptible breakpoint (μg/mL)	Resistant breakpoint (μg/mL)
ampicillin	≤8	≥32
ciprofloxacin	≤0.06	≥1
tetracycline	≤4	≥16

3.4 On-chip test with real food samples

3.4.1 Spiked sample preparation

We purchased raw, skinless, boneless chicken breast products for the tests. According to the recommendations from U.S. Food and Drug Administration BAM protocol. The chicken breast meat were cut into 25-g pieces (Center for Food Safety and Applied, 2021, 2022). To eliminate other species that were not tested in this project and control the initial concentration of *Campylobacter* and *Salmonella*, 1% sodium hypochlorite aqueous solution (Sigma-Aldrich, USA) was applied to disinfect the chicken meat surface. After surface sterilization, defined concentration

of *Campylobacter* or *Salmonella* ($10^2 - 10^8$CFU/25 g) was spiked onto the chicken sample, and the spiked meat was air dried in the biosafety cabinet.

We then tested the recovery. The spiked chicken samples were placed into a pre-sterilized stomach bag. PBS (25 mL) was added into the bag and chicken samples were hand massaged for 3 minutes. The rinse solution was collected and the wash-off tissue particles were removed by being filtered through a pre-sterilized Whatman No.1 filtration paper. Then, the filtered suspension was loaded into the microfluidic chip for the tests.

3.4.2 Sensitivity test

To test the limit of detection (LOD) of this microfluidic chip using real food samples, a range of concentrations of *C. jejuni* F38011 and *S.* Enteritidis 43353 ($10^2 - 10^8$CFU/25 g) were injected and incubated at 42°C for 60 hours under the microaerobic environment. Mixed culture was used in this test. For sensitivity test of *Campylobacter*, the background culture was *S.* Enteritidis 43353 (10^5 CFU/25g). For sensitivity test of *Salmonella*, the background culture was *C.* jejuni F38011 (10^8 CFU/25g). The chips were monitored with a time period of 12 hours for up to 60 hours. Aside from the spiked chicken samples, non-spiked chicken samples were used as the negative control and went through the same procedure.

3.4.3 Antimicrobial susceptibility testing (AST)

The procedure of AST was the same as the previous tests as aforementioned. The used bacterial culture was extracted from the spiked chicken samples. Taken the effects of recovery rate and dilution effects from the addition of PBS into consideration, the initial spiked concentrations were increased by centrifugation (5×10^{10}CFU/25 g for *Campylobacter*, 5×10^7CFU/25 g for *Salmonella*) to allow the final recovered concentration to reach to the standard concentrations for AST (10^8CFU/mL for *Campylobacter* and 10^5CFU/mL for *Salmonella*). The recovery rate in this study was ~10%.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Interpretation of microfluidic chip design

In this study, a microfluidic lab-on-a-chip device was developed for identification and AST of *Campylobacter* and *Salmonella*. This chip was composed by two layers of poly(dimethylsiloxane) (PDMS) slabs and one layer of a glass slide. The assembly of three layers is described in **Figure 4.1 (a).** *Campylobacter* and *Salmonella* were spiked onto the chicken sample and recovered. The rinsed solution was then injected into the chip. After incubation, the results were interpreted based on color. Within the PDMS-based chamber layer, there were 14 incubation chambers that were located separately and connected to a main channel which locates in the middle of the chip via side channels. Incubation chamber and the main channel were connected by the side channel that had a zigzag-shaped for the prevention of the backflow of sample fluids into the adjacent incubation chambers. PDMS-based injection layer consists air vent beside every incubation chamber. During the sample injection, the air in each incubation chamber was removed out through air vents to release the internal pressure and give loading space for bacterial suspension in the incubation chambers.

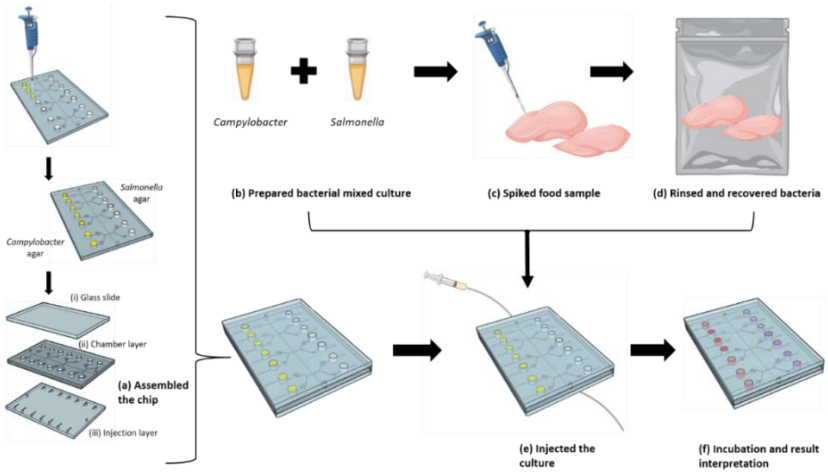


Figure 4.1 Schematic illustration of the process of microfluidic chip assembling and its application for dual-species detection. (a)*Campylobacter* chromogenic agar, *Salmonella* chromogenic agar, and antimicrobials were loaded into the microfluidic chip. An injection layer (i), a chamber layer (ii), and a glass slide (iii) were bonded together using a plasma cleaner. (b) *Campylobacter* and *Salmonella* cultures were both (c) spiked onto the pre-sterilized chicken breast sample. (d) The chicken breast samples were transferred into a stomach sampling bag to recover bacteria using

phosphate-buffered saline (PBS). (e) PBS was later injected into the prepared chip for (f) incubation.

After the assembling, the entire chip was 50 mm in length × 37 mm in width × 6 mm in height. The sample injection setting is shown in **Figure 3.2**. The pump-driven force was provided by an external pump with a flow rate of 50 µL/min. After the main channel was filled with bacterial suspension, a binder clip was used to block the outlet tubing to allow the resistance in the main channel to increase until it exceeded the pressure in the side channels. This could facilitate bacterial sample to distribute into the incubation chambers.

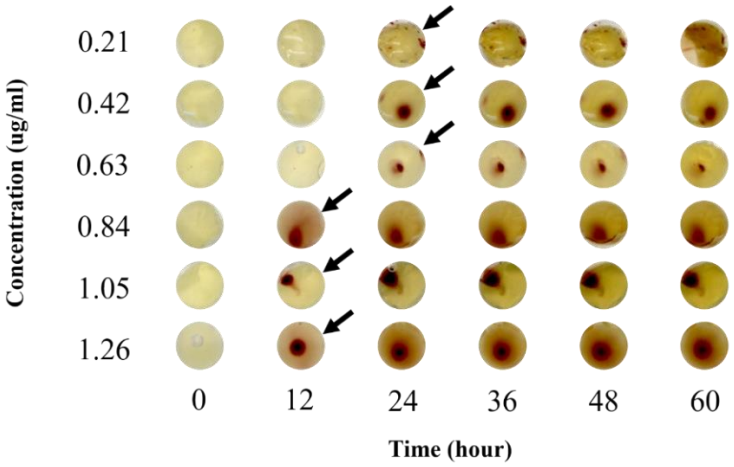
The microfluidic chip could carry out identification test and investigate bacterial susceptibility towards different classes of antimicrobials simultaneously. The antimicrobials could diffuse from the paper discs into the agar during the incubation step (Cira et al., 2012). In this case, different classes and/or concentrations of antimicrobials were prevented from cross-contamination by the zigzag-shaped channels (Ma et al., 2020). Diffusion test was conducted to verify if the microfluidic chip was free of cross-contamination between incubation chambers (**Figure 3.3**). No color alteration was observed in any incubation chamber, which validated that the design of the microfluidic chip could provide an effective function to prevent cross-contaminations among incubation chambers.

4.2 Optimization of chromogenic agar

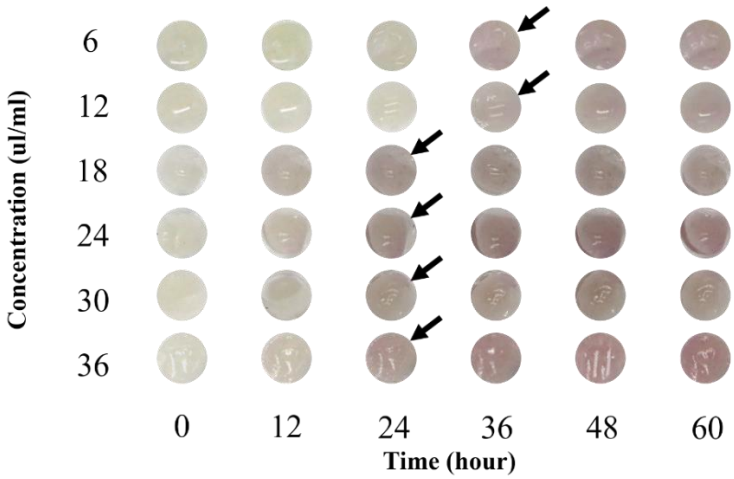
The optimal concentration test of chromogenic substrates was carried out for enhancing the visual detection of the chromogenic reactions. Six different supplement concentrations of each chromogenic agar were chosen to prepare the chip to carry out optimization tests. For *Campylobacter*, these were 0.21, 0.42, 0.63, 0.84, 1.05, and 1.26 µg/mL. For *Salmonella*, they were 6, 12, 18, 24, 30, and 36 µL/mL. The supplement concentration could offer a relatively quicker color change and chosen to be the supplement concentration in the later experiments. For *Campylobacter* chromogenic agar and *Salmonella* chromogenic agar, a mixture with a final concentration of *C. jejuni* F38011 (10^8CFU/mL) and *S.* Enteritidis 43353 (10^5CFU/mL) was injected and incubated in the microfluidic chips at 42°C for 60 h. During the incubation, the

existence of red color pigments or mauve color pigments indicate the time points when visible color changes could be observed. Images were collected with a time interval of 12 hours by using a Canon camera (model of EOS Rebel T6i) at each time point.

For *Campylobacter* chromogenic agar, the recommended concentration by the company is 0.21 µg/mL, but the optimal concentration is 0.84 µg/mL that could give a quicker response at 12 hours. Tested samples with 1.05 and 1.26 µg/mL could also give color change at this time point, but 0.84 µg/mL was still chosen at last due to cost consideration. For *Salmonella* chromogenic agar, the recommended concentration by the company is 6 µl/mL and the final selected optimal concentration was 18 µL/mL. The selection rationale is still based on response time and lowest possible concentration.



(a)



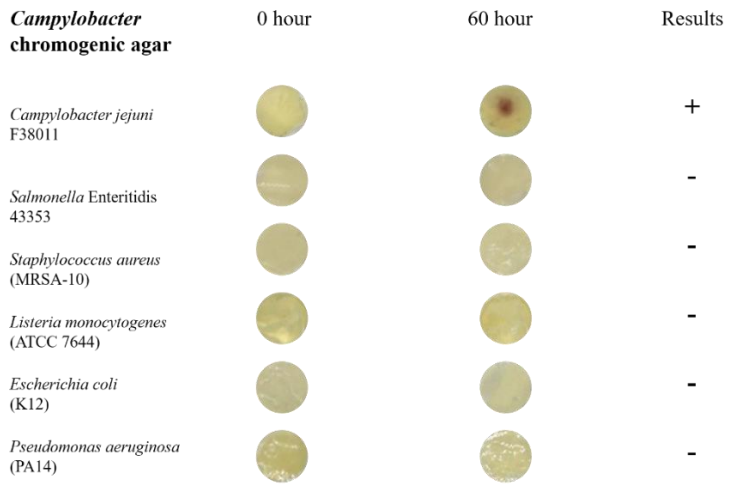
(b)

Figure 4.2 Optimal supplement test of (a) *Campylobacter* chromogenic agar and (b) *Salmonella* chromogenic agar. Representative images of (a) *C. jejuni* F38011 on-chip detection and (b) *S.* Enteritidis 43353 on-chip detection. Six different supplement concentrations of each chromogenic agar were chosen to prepare the chip to carry out the optimization tests. The supplement concentration that can offer a relatively quicker color change was chosen to be the supplement concentration in the later experiments. For *Campylobacter* chromogenic agar, *C. jejuni* F38011 with a final concentration of 10^8 CFU/mL was injected and incubated in the microfluidic chips at 42°C for 60 h. Simultaneously, *S.* Enteritidis 43353 with a final concentration of 10^5 CFU/mL was mixed as a background culture to mimic the scenario of spiked food sample with multiple bacteria. During the incubation, the existence of red color pigments indicates the time points when visible color changes can be observed. For *Salmonella* chromogenic agar, *S.* Enteritidis 43353 with a final concentration of 10^5 CFU/mL was injected and incubated in the microfluidic chips at 42°C for 60 h. Simultaneously, *C. jejuni* F38011 with a final concentration of 10^8 CFU/mL was mixed as a background culture to mimic the scenario of spiked food sample with multiple bacteria. During the incubation, the existence of mauve color pigments indicate the time points when visible color changes could be observed. The visible color changes are indicated by arrows. These concentrations were chosen according to the standards of antimicrobial susceptibility testing in CLSI protocol (M07-A9, Vol. 32 No. 2, M45) (PA, 2016, 2020).

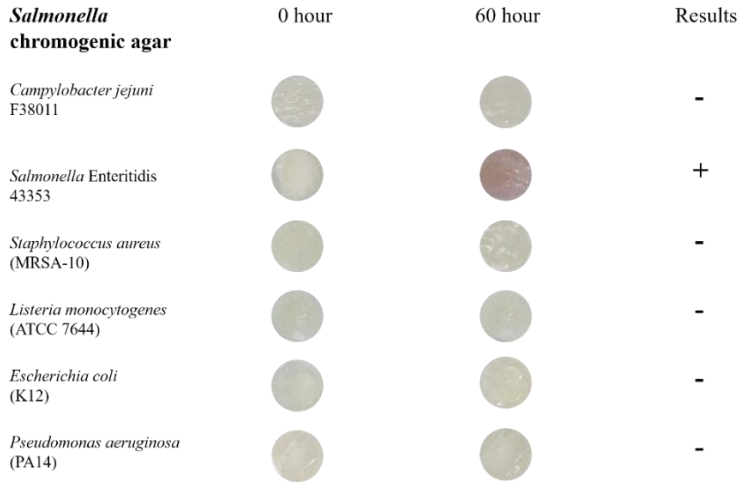
4.3 On-chip identification test with pure culture

The specificity and sensitivity to the targeted bacteria are critical criteria to evaluate the performance of detection methods. *Campylobacter*, *Salmonella*, *Staphylococcus, Listeria monocytogens, Escherichia coli, Pseudomonas aeruginosa* were loaded onto both *Campylobacter* chromogenic agar (**Figure 4.3 a**) and *Salmonella* chromogenic agar (**Figure 4.3 b**) at the final concentration: 10^8 CFU/mL. They were incubated in the microfluidic chips at 42°C for 60 h under microaerobic conditions. No color change was observed in *Campylobacter* chromogenic agar loaded with non-*Campylobacter* species or *Salmonella* chromogenic agar loaded with non-*Salmonella* species. Red signals and mauve signals were observed when these two chromogenic agars encounter the target bacteria. This indicates that the developed assay can be used to distinguish *Campylobacter* and *Salmonella* when they are in the same mixture. The color intensity

and the shape of the pigment area may vary from chamber to chamber. Positive results were determined based on the existence of red or mauve signals as a yes or no result. All in all, on-chip identification was specific to the two selected bacterial species.



(a)

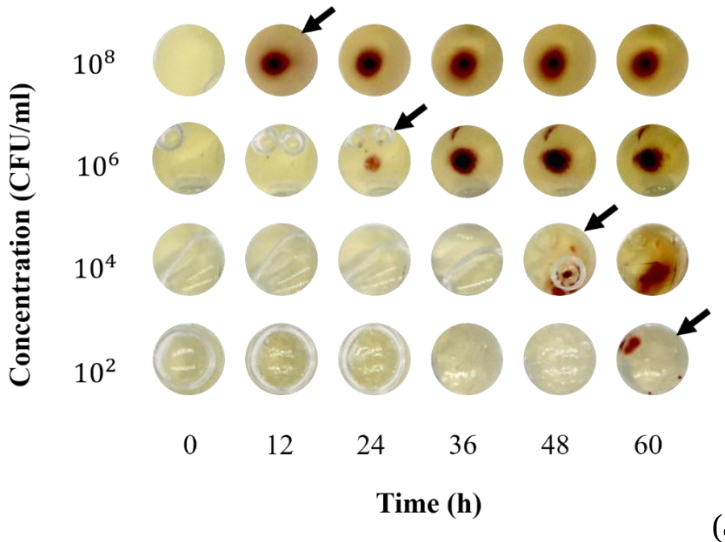


(b)

Figure 4.3 Specificity test. *Campylobacter*, *Salmonella*, and other bacteria species (10^8CFU/mL) were loaded onto both *Campylobacter* chromogenic agar (a) and *Salmonella* chromogenic agar (b). They were injected and incubated in the microfluidic chips at 42°C for 60 h. The tested bacterial strains included *C.jejuni* F38011, *S.* Enteritidis 43353, *Staphylococcus aureus* MRSA-10, *Listeria monocytogens* ATCC 7644, *Escherichia coli* K12, *Pseudomonas aeruginosa* PA14. In (a) and (b),

the chromogenic agar did not have any color change for the non-target bacteria while it showed color change with red and mauve when encountered the target bacteria.

In sensitivity test, a range of concentrations of *C. jejuni* F38011 and *S.* Enteritidis 43353 (10^2, 10^4, 10^6, 10^8 CFU/mL) were cultivated in MHB or LB for on-chip detection. When different concentrations of these two bacteria culture tested, the other bacterium was prepared as a background culture with 10^8 CFU/mL (*Campylobacter*) or 10^5 CFU/mL (*Salmonella*) and mixed with the tested bacteria. Red signals or mauve signals represent positive results regardless of color intensities. The generated color from bacterial metabolism in the chromogenic-agar-based microfluidic chips could be easily recognized by the naked eyes. For *Campylobacter* chromogenic agar, with a high initial load of bacteria (e.g. 10^8 CFU/mL), the red signals could appear at 12-hour time point. Along with reduced concentration, the response time extended gradually. The lowest detection limit was 10^2 CFU/mL within a 60-hour incubation period, which was shorter than the conventional isolation procedures (48-72 hour) (Government of Canada, 2018). For *Salmonella* chromogenic agar, the highest concentration chosen for sensitivity test also showed color change at 12-hour time point. For the other three selected concentrations, the color signals appeared faster than the same tested concentrations of *Campylobacter*. This is due to the more rapid growth rate of *Salmonella* than *Campylobacter* (Battersby et al., 2016; Shands, 1965).



(a)

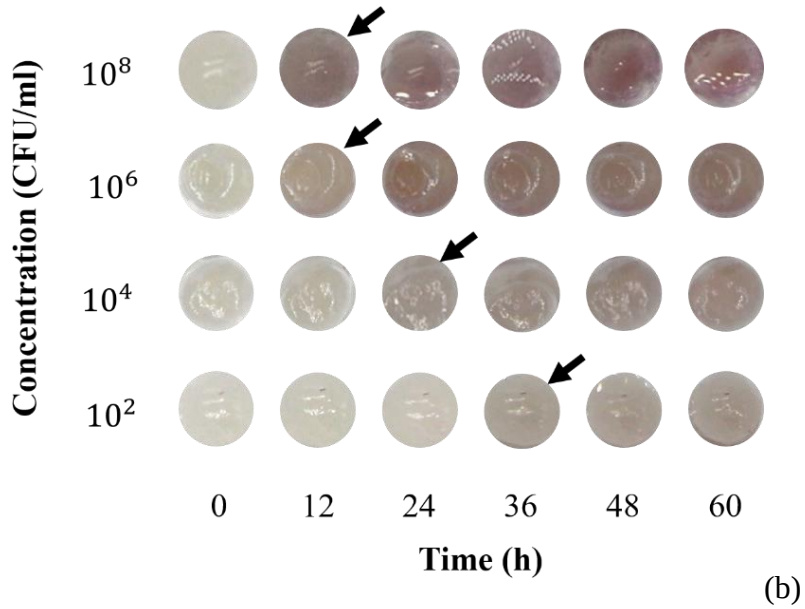


Figure 4.4 Limit of detection test. Representative images of (a) *C. jejuni* F38011 on-chip detection and (b) *S.* Enteritidis 43353 on-chip detection. Four different concentrations of bacteria culture were chosen to test the sensitivity of the chip. *C. jejuni* F38011 with a concentration of 10^2, 10^4, 10^6, 10^8 CFU/mLwas mixed with *S.* Enteritidis 43353 with a concentration of 10^5 CFU/mL, and injected and incubated in the microfluidic chips at 42°C for 60 h. A red color pigment indicates the time points when visible color changes can be observed. *S.* Enteritidis 43353 with a concentration of 10^2, 10^4, 10^6, 10^8 CFU/mLwas mixed with *C. jejuni* F38011 with a concentration of 10^8CFU/mL, and injected and incubated in the microfluidic chips at 42°C for 60 h. A mauve color pigment indicates the time points when visible color changes can be observed. The visible color changes are indicated by arrows.

4.4 On-chip Antimicrobial susceptibility test (AST) with pure culture

The principle of on-chip AST follows the conventional broth microdilution method. For every individual incubation chamber, a defined amount of antibiotics with pre-determined concentration was pre-loaded onto a sterilized paper disc, followed by the loading of chromogenic agar. Then, the bacterial culture was injected into each incubation chamber with the same amount as the chromogenic agar. This step mimics the 2-fold dilution in broth microdilution. The final

adjusted concentrations of antibiotics were the same as the CLSI breakpoints. During bacterial cultivation, the antibiotics were expected to diffuse from paper discs to the chromogenic agar. The resistance ability of the resistant bacteria were identified if there was color change. The presence of the color indicates bacterial growth, which means that bacteria are resistant to an antibiotic with a defined level of concentration. In the case of no color change, the bacteria are susceptible to that antibiotic.

The multi-drug resistance (MDR) in *Campylobacter* and *Salmonella* is a serious threat to public health (Castro-Vargas et al., 2020; Centers for Disease Control and Prevention, 2019). MDR was defined as that target bacteria are resistant to three or more antimicrobial classes. Agri-food system needs efficient and cost-effective tests for determining bacterial multi-drug susceptibility. Therefore, we developed on-chip multi-drug AST that follows the susceptibility criteria from the official documents published by the Clinical and Laboratory Standards Institute (CLSI). Other than having a series of antibiotic concentrations to test the exact MIC values, the on-chip MDR test involved only two breakpoints which were 'susceptibility' and 'resistance' by CLSI (PA, 2016, 2020). This microfluidic chip can give three different readings on the bacterial antimicrobial susceptibility: (1) "susceptible" strains represented those strains that did not change agar color at both susceptibility and resistance breakpoints; (2) "non-susceptible" strains were determined when color alteration was generated at susceptibility breakpoint but no color alteration at resistance breakpoint; (3) "resistant" strains produced color on agar at susceptibility and resistance breakpoints.

Four different antibiotics were selected for the later AST, namely ampicillin, ciprofloxacin, erythromycin, and tetracycline.

For *Campylobacter*, the tested antibiotics were ciprofloxacin, erythromycin, and tetracycline. The most common drugs to which *Campylobacter* is resistant include fluoroquinolones (FQ), macrolides, tetracycline, florfenicol, trimethoprim, and sulfamethoxazole (Szczepanska et al., 2017; Li et al., 2017; Ma et al., 2017). FQs are broad-spectrum antimicrobials and are applied in situations with multitude of infections, predominantly by using ciprofloxacin. Ciprofloxacin can be considered as the first-defense line for campylobacteriosis (Sproston et al., 2018). The FQ-antimicrobial resistance in *Campylobacter* species has been initially reported in the late 1980s in Europe (Engberg et al., 2001). Globally, a steep increasing trend in FQ-resistant

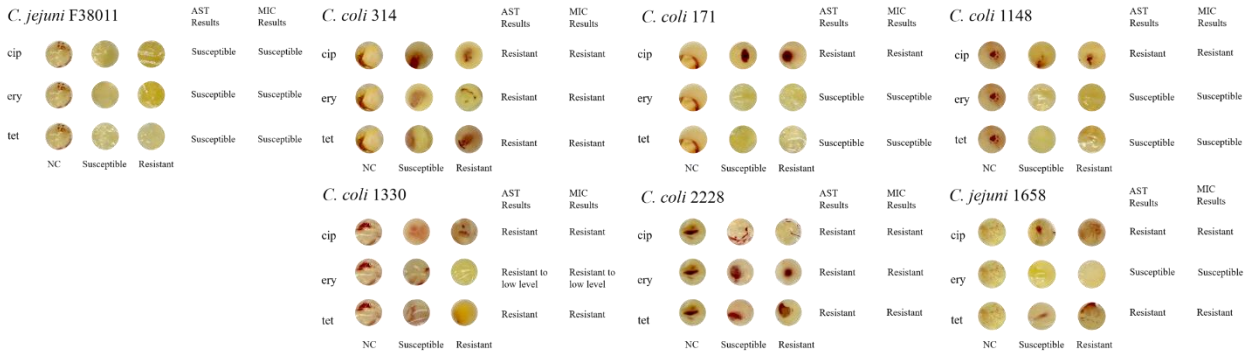
Campylobacter incidence has been reported worldwide (Luangtongkum et al., 2009; Nguyen et al., 2016; Padungton and Kaneene, 2003; Sierra-Arguello et al., 2016; Woźniak-Biel et al., 2018). Although controlling respiratory diseases was the prior reason for using FQs intentionally instead of controlling campylobacteriosis. The usage of FQs was correlated with the FQ-resistance in *Campylobacter* (Luo et al., 2003; McDermott et al., 2002; Zhang et al., 2003). A study reported that in Thailand, there was no ciprofloxacin resistance of *Campylobacter* species in humans before 1991, but it increased to 84% in 1995 (Hoge et al., 1998). A 17-year study of clinical C. jejuni isolated in China had the similar result that the resistance rate increased from 50% to 93.1% (1994-2010) (Zhou et al., 2016). Aside from Asia, many European countries has also reported increasing incidence in FQ-resistance among *Campylobacter* isolates (Gallay et al., 2007; Lucey et al., 2002; Pezzotti et al., 2003). Tetracyclines also belong to broad-spectrum antibiotics that are used in the management and treatment of infectious diseases (Shutter & Akhondi, 2019). According to the study in Thailand, 98.9% of *C. coli* isolates and 100% of *C. jejuni* isolates from commercial broiler production chains were confirmed to be MDR. Most of them were resistant to FQ, tetracycline, and trimethoprim (Thomrongsuwannakij et al., 2017). Several other studies showed that the most common antibiotics from MDR pattern in the USA were ciprofloxacin, nalidixic acid, and tetracycline (Benoit et al., 2014, pp. 2008–201; Ricotta et al., 2014, pp. 2005–2011). In this sense, these two antibiotics were selected to mimic the practical scenario when they were prescribed by a professional for treatment at the first place.

In comparison with FQ class, resistance to macrolides is much less prevalent in *Campylobacter* so that macrolides can be used to treat campylobacteriosis. In this class, erythromycin has been used frequently (Sproston et al., 2018). Its resistance is usually lower than 10% in most developed countries (Cha et al., 2016; Engberg et al., 2001). The National Animal Health Monitoring System (NAHMS) Dairy 2002 and Dairy 2007 reported that 0.4% *Campylobacter* isolates from cattle had resistance to erythromycin. In NAHMS 2014 report, both human and chicken *C. jejuni* isolates were reported with a resistance to erythromycin which was less than 2% while *C. coli* isolates was around 10% (USDA, 2011). Erythromycin was selected as the next defense towards the bacterial strains which are resistant to the first-defense antibiotics.

For *Salmonella*, the tested antibiotics were ampicillin, ciprofloxacin, and tetracycline. Ampicillin is one of the first-line antibiotics for treating salmonellosis (Crump et al., 2015). In the

study carried out on antibiotic resistance of *Salmonella* in poultry, ampicillin and nalidixic acid were the antibiotics that bacteria had the highest resistance levels within the poultry production chain (Castro-Vargas et al., 2020). Tetracyclines are broad-spectrum antimicrobial drugs that are used to against a wide range of infections introduced by both Gram-positive and Gram-negative bacteria (McManus et al., 2002). This antibiotic is the current choice for treatment, but the resistance of bacteria found in humans and animals increases gradually (Frech & Schwarz, 2000). The fluoroquinolone (FQ) ciprofloxacin is one of the recommended drugs to treat salmonellosis. Although the resistance towards ciprofloxacin has increased a little, the efficacy of this antibiotic did not change significantly. According to NARMS reports, the percentage of non-susceptible *Salmonella* isolates increased from <0.5% up to 3.5% since 1996 (Shane et al., 2017). In 2015, the EUCAST (European Committee on Antimicrobial Susceptibility Testing) database showed that total *Salmonella* isolates that were non-susceptible to ciprofloxacin was 6% (European Committee on Antimicrobial Susceptibility testing, 2015). Overall, ampicillin and tetracycline were selected as the first treatment option while ciprofloxacin was the alternative choice.

Different results can be categorized by on-chip MDR tests (**Figure 4.5**). The negative-control chamber was the chamber where there was no antibiotics added into the loaded chromogenic agar. The existence of red signals and mauve signals indicated that the device can provide an appropriate cultivation environment and the bacteria can grow normally inside this chamber. For validation, conventional minimum inhibitory concentration (MIC) tests were carried out. The results were shown in. Two susceptible strains were also tested as comparison groups.



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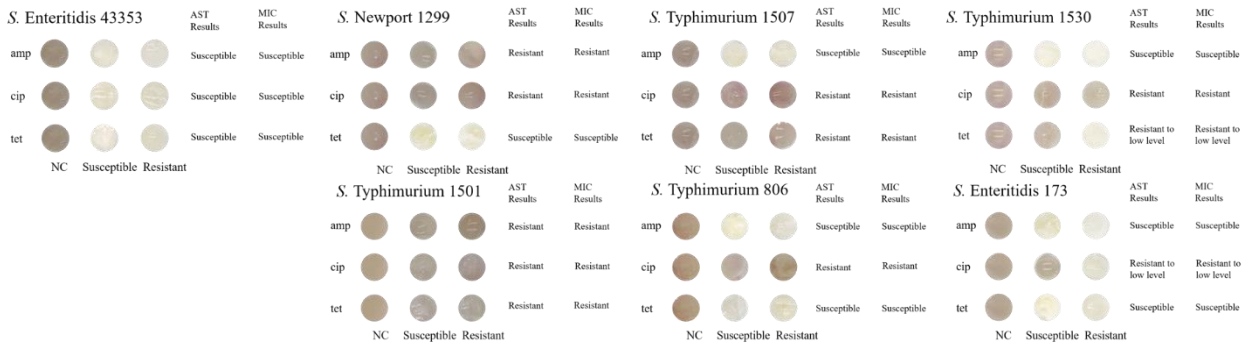


(b)

Figure 4.5 On-chip antimicrobial susceptibility testing (AST) to determine multiple drug resistance profiles of *C. coli* 171, *C. coli* 314, *C. coli* 1148, *C. coli* 1330, *C. jejuni* 1658, *C. coli* RM2228, *S.* Enteritidis S173, *S.* Typhimurium S806, *S.* Newport S1299, *S.* Typhimurium S1501, *S.* Typhimurium S1507, *S.* Typhimurium S1530. Each chamber is deposited with selected types of antibiotics, ciprofloxacin (cip), erythromycin (ery) tetracycline (tet), and ampicillin (amp) at different concentration levels which are resistant-level concentration (r) and susceptible-level concentration (s). The chamber loaded with agar without antibiotic is the negative control (NC). Each row represents a level of concentrations for antibiotics, as follows: NC, no antibiotic; susceptible, CLSI breakpoints for susceptible strains; resistant, CLSI breakpoints for resistant strains. The concentrations of each antibiotic for *Campylobacter* spp. are listed as follows: (i) for ciprofloxacin, the two concentrations were 1 and 4 ug/mL, respectively; (ii) for erythromycin, the two concentrations were 8 and 32 ug/mL, respectively; (iii) for tetracycline, the two concentrations were 4 and 16 ug/mL, respectively.(PA, 2016) The concentrations of each antibiotic for *Salmonella* spp. are listed as follows: (i) for ampicillin, the two concentrations were 8 and 32 ug/mL, respectively. (ii) for ciprofloxacin, the two concentrations were 0.06 and 1 ug/mL, respectively; (iii) for tetracycline, the two concentrations were 4 and 16 ug/mL, respectively.(PA, 2020)

The categorical agreement rates between the conventional MIC tests and on-chip AST were calculated for verification of the accuracy.The categorical agreement rate is defined as the percentage of results obtained by a newly-established method matches with the results from reference methods (Humphries et al., 2018). A total of 7 *Campylobacter* strains and *Salmonella* strains were tested, and results were listed in **Figure 4.5**. Aside from all the antimicrobial-resistant strains, two antibiotic-susceptible strains (*C. jejuni* F30811 and *S.* Enteritidis 43353) have also

been tested. The final agreement rates are 96.76% for *Salmonella* species and 95% for *Campylobacter* species (**Table 4.1**). According to U.S. Food and Drug Administration, it requires a value above 90% as the acceptable categorical agreement rate. In this case, our results meet this requirement.

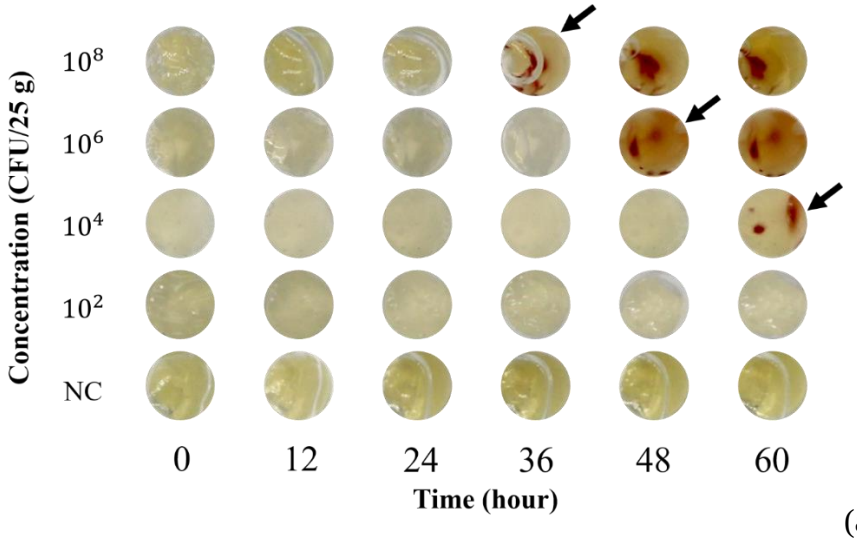
Table 4.1 Categorical agreement rate for *Salmonella* and *Campylobacter*

Bacteria species	**AST results (correct/total)**			**Categorical agreement rate (%)**
Salmonella	***S.* Newport**	***S.* Typhimurium**	***S.* Enteritidis**	
	100%	95.83%	94.44%	96.76%
Campylobacter	***C. coli***	***C. jejuni***		
	95.56%	94.44%		95%

4.5 Sensitivity test with food samples

Poultry is a common food source for both *Campylobacter* and *Salmonella* (Hugas & Beloeil, 2014; Skirrow, 1977). To better understand the performance of this microfluidic chip with the involvement of actual food products, raw chicken samples were applied to carry out on-chip tests. For sensitivity test for *Campylobacter* chromogenic agar, *C. jejuni* F38011 (a) and *C. coli* RM1875 (b) were used to spike onto the chicken samples with the initial bacteria load to be 10^2, 10^4, 10^6, 10^8 CFU/25 g. *S.* Enteritidis 43353 with a concentration of 10^5 CFU/25 g was spiked as a background culture. Both two species grew on the agar and generated red signals in the microfluidic chip. As for *Salmonella* chromogenic agar, *S.* Enteritidis 43353 (c) and *S.* Typhimurium S1501 (d) were chosen. Their initial loads were 10^2, 10^4, 10^6, 10^8 CFU/25 g. The background culture was *C. jejuni* F38011 culture with a concentration of 10^8 CFU/25 g. Mauve signals were found in the microfluidic chip for both species. In all the negative-control chip, the rinse solution from pre-sterilized chicken samples did not give visible color alteration to both agars

throughout the entire incubation. This test proved the selectivity of this device for bacterial detection. Both limit of detection of Campylobacter and Salmonella were 10^4 CFU/25 g. Detection for *Campylobacter* and *Salmonella* in chicken meat experienced certain delays compared with the tests using the pure bacterial culture. The increasing in their turnaround time and reduced sensitivity in testing actual food sample were caused by dilution from the recovery step using PBS.



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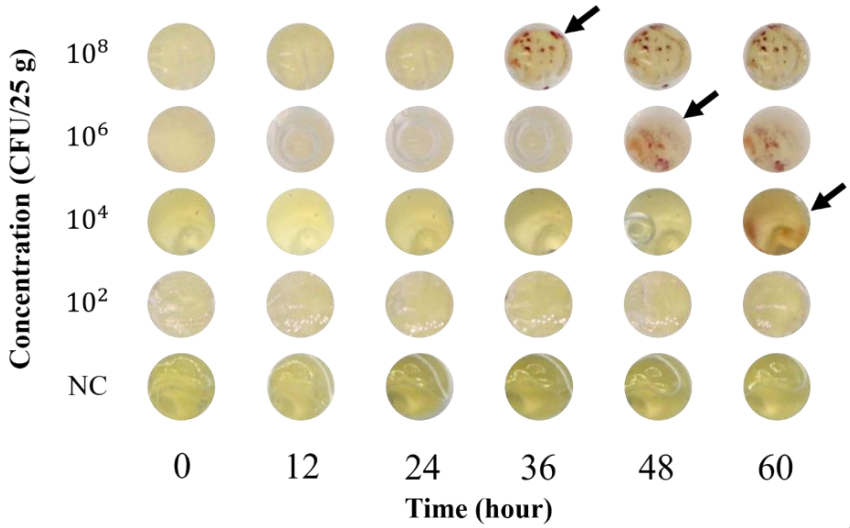

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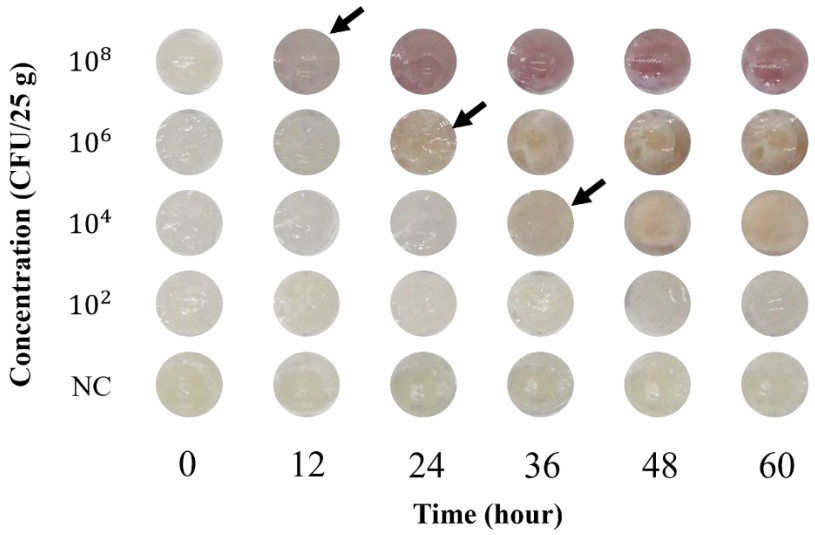

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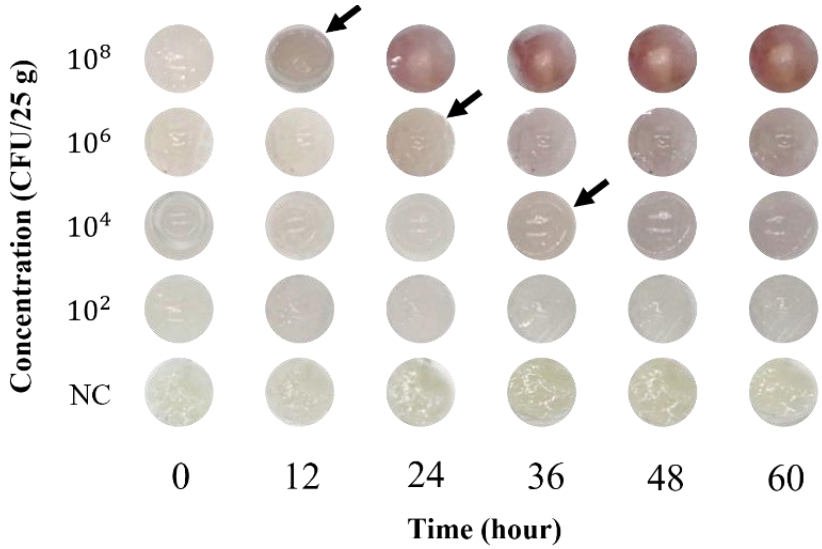

(d)

Figure 4.6 Detection of bacteria in fresh chicken meat by using the microfluidic chip. Four different concentrations (10^2, 10^4, 10^6, 10^8 CFU/25 g) of bacteria culture were chosen to be spiked onto the chicken sample and to test the sensitivity of the chip. The bacteria will be extracted by 25 ml of phosphate-buffered saline (PBS) solution, and the mixture was injected and incubated in the microfluidic chips at 42°C for 60 h. The existence of a color pigment indicates the time points when visible color changes can be observed. The tested *Campylobacter* species are *C. jejuni* F38011 (a) and *C. coli* RM1875 (b). *S.* Enteritidis 43353 culture with a concentration of 10^5 CFU/25 g was spiked as a background culture to mimic the actual scenario. The tested *Salmonella*

species are *S.* Enteritidis 43353 (c) and *S.* Typhimurium S1501 (d). *C. jejuni* F38011 culture with a concentration of 10^8 CFU/25 g was spiked as a background culture to mimic the actual scenario. The visible color changes are indicated by arrows.

4.6 Antimicrobial susceptibility test (AST) with food samples

To obtain a final concentration that meets the standard concentrations for AST, the initial spiked concentrations were adjusted to 5×10^{10} CFU/25 g for *Campylobacter* and 5×10^7 CFU/25 g for *Salmonella*. The procedure of the AST was the same as the previous tests. The final results obtained in on-chip AST matches the results from MIC tests. (**Figure 4.5**) However, to achieve a proper concentration that matches the AST standards, the spiked concentration needs to be very high. This concentration may exceed the normal bacteria load in the practical situation. In this case, the extract bacteria suspension may undergo enrichment step to allow the concentration reach the desired amount before being put into the tests.

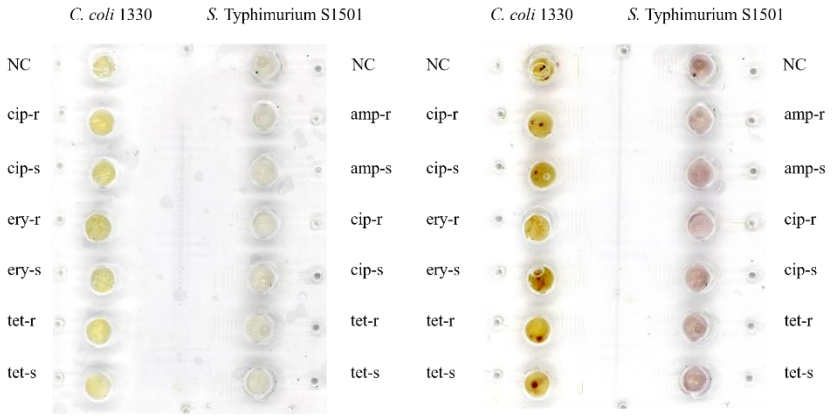

Figure 4.7 An example of on-chip antimicrobial susceptibility testing (AST) to determine multiple drug resistance profiles of *C. coli* 1330 and *S.* Typhimurium S1501 extracted from chicken sample. The spiked concentration is 5×10^{10} CFU/25 g for *C. coli* 1330 and 5×10^7 CFU/25 g for *S.* Typhimurium S1501. The spiked concentration was increased to overcome the dilution effect from 25 ml of PBS solution. Image (a) is the chip at 0 hour while image (b) is the chip after incubated for 24 hours. *C. coli* 1330 growth can be inhibited by resistant level of erythromycin while *S.* Typhimurium S1501 is resistant to all the levels of selected antibiotics. Each chamber is deposited with selected types of antibiotics, ciprofloxacin (cip), erythromycin (ery) tetracycline (tet), and

ampicillin (amp) at different concentration levels which are resistant-level concentration (r) and susceptible-level concentration (s). The chamber loaded with agar without antibiotic is the negative control (NC). Each row represents different concentrations of antibiotics, as follows: NC, no antibiotic; susceptible, CLSI breakpoints for susceptible strains; resistant, CLSI breakpoints for resistant strains.

CHAPTER 5. CONCLUSION

In this study, a hybrid microfluidic device was developed to rapidly identify and carry out antimicrobial susceptibility test (AST). The microfluidic chip was made of PDMS and paper to keep the liquid suspension inside the system as well as facilitate the antimicrobial loading. The designed patterns could be fabricated successfully using the silicon master. In addition, the microfluidic chip could function normally and prevent cross-contamination from the adjcent incubation chambers. The on-chip test results were shown based on the colorimetric reaction. To achieve this, chromogenic agar was loaded into the microfluidic chip to selectively cultivate and differentiate bacterial species. The optimization test confirmed the optimal supplement concentration for the chromogenic agar to be 0.84 μg/mL for *Campylobacter* chromogenic agar and 18 μL/mL for *Salmonella* chromogenic agar. These two agars had good specificity towards the target bacteria and did not show any color change to other species. The LOD of this microfluidic chip was 10^2 CFU/mL for pure bacterial culture. On-chip AST results using pure bacterial culture matched with that by the conventional broth micro-dilution method with a categorical agreement rate >90%. In the case of using spiked chicken sample, the LOD was 10^4 CFU/25 g due to the dilution effect during the extraction step.

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