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# Development of biosensor-based assays to identify anti-infective oligosaccharides

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ABSTRACT

It is now well accepted that milk oligosaccharides can have a direct inhibitory effect on pathogenic microorganisms by interfering with their adhesion to human cells. Many free oligosaccharides from milk are considered to be soluble receptor analogs of epithelial cell surface carbohydrates and, thus, function as receptor decoys to which pathogens can bind instead of the host. In reality, there are few rapid methods to screen for such oligosaccharides, and much of the research in this area has centered on using human cell line models of infection that are time-consuming. Therefore, a quick and sensitive method is required for detecting the binding of microorganisms to milk oligosaccharides. Our study describes a number of biosensor-based methods to achieve these aims. Our approach involved the exposure of whole bacterial cells to the well-characterized human milk oligosaccharide, 2′-fucosyllactose, immobilized to a pretreated gold chip surface. The technique was validated by screening a range of pathogenic bacteria, including *Campylobacter jejuni,* to which 2′-fucosyllactose is known to bind. Where binding was detected, its specificity was confirmed by preincubation studies using unlabeled 2′-fucosyllactose. The techniques described represent a quick, cost-effective, and highly reproducible detection method for identifying anti-infective oligosaccharides.



Adherence is the first step in the pathogenic cycle of invading bacteria. Over evolutionary time, pathogenic bacteria have evolved a multitude of adhesion mechanisms that commonly target carbohydrate structures on the host cell surface. Many free oligosaccharides and glycoproteins from milk have been shown to act as soluble receptor analogs of epithelial cell surface carbohydrates [1]. These oligosaccharides display structural homology to host cell receptors and, thus, function as receptor decoys. Milk oligosaccharides can also inhibit pathogens by competitive binding to host cell surface receptors [2]. The use of oligosaccharides sourced from foods, or bioidentical versions of them, is an attractive approach to prevent pathogen colonization because the regulatory hurdles to their use are substantially less than for other compounds. Food-sourced oligosaccharides can, therefore, be seen as ideal candidates for inclusion in functional foods. In addition, their use may be safer than conventional antibiotics because they may be less prone to the generation of abnormal selection pressures in microbial populations at mucosal surfaces [3]. There are a number of studies describing the adherence of pathogenic bacteria to neutral and acidic milk oligosaccharides. For example, *Campylobacter jejuni,* one of the most predominant causes of diarrhea worldwide, adheres to intestinal 2′-fucosyllactosamine, which is also present on certain human milk oligosaccharides (HMOs).[1] Furthermore, fucosylated HMOs inhibit *Campylobacter* binding to human intestinal mucosa *ex vivo*, and the incidence of *Campylobacter* diarrhea in breast-fed infants is inversely related to the amount of 2′-fucosyllactose in the mother's milk [4–6]. Anti-infective effects of HMOs were also described for *Helicobacter pylori, Salmonella fyris, Pseudomonas aeruginosa,* calicivirus, and the heat-stable enterotoxin of *Escherichia coli* [4,7–9]. Conventional tissue culture-based screening methods used for monitoring the ability of milk oligosaccharides to prevent adhesion of bacteria to the host cell are time-consuming and do not provide information on binding dynamics in real time.

Biomolecular interaction analysis (Biacore) involves the use of surface plasmon resonance (SPR) to measure the binding of candidate compounds or cells to specific ligands [10]. SPR measures the changes in refractive index near a planar chip surface induced by binding of soluble molecules to immobilized counterpart molecules on the sensor chip [11]. It has been widely used for the quantification and kinetic analysis of receptor–ligand interactions [12,13], but to date the number of studies using SPR for the characterization of bacterial cell interactions is limited [14–17]. Besides being a quantitative method, SPR measures interactions

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[1] *Abbreviations used:* HMO, human milk oligosaccharide; SPR, surface plasmon resonance; EDTA, ethylenediaminetetraacetic acid; CFU, colony-forming units; NHS, N-hydroxysuccinimide; EDC, *N*-ethyl-*N*-(dimethyl-aminopropyl)carbodiimide hydrochloride; RU, response units; ssDNA, single-stranded DNA.

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dynamically as a function of time in a continuous flow system. Therefore, SPR assays may mimic natural conditions in a more realistic manner than static adhesion assays because the main purpose of microbial adhesion is to prevent bacteria from detaching from their target surface under vigorous shear stresses such as those found in blood vessels and various luminal organs lined by mucus membranes [18]. This study uses 2′-fucosyllactose as a model target to validate the use of SPR for screening bacterial binding to milk oligosaccharides. It exploits the known interaction between *C. jejuni* and this HMO to obtain "proof of concept" for this approach. A number of Biacore approaches, both classical and newly developed, were explored in the search for the most sensitive and highly reproducible model system. An EZ-Link biotin–$PEG_4$–hydrazide was attached to the reducing end of 2'-fucosyllactose to allow immobilization to the chip surface directly or indirectly through the use of an anti-biotin antibody. The classic CM5 and SA Biacore chips were initially used in our study. There are a number of limitations when using these chips. Stringent optimization is often required when using the CM5 chip, with regeneration conditions being a constant stumbling block that can directly alter the binding capacity of the chip surface. In addition, the use of an SA chip for the reversible capture of biotin molecules is extremely difficult due to the strong interaction between streptavidin and biotin. There is a need for a highly reproducible, easily regenerated, and relatively sensitive chip surface. Therefore, the use of the newly developed Biacore biotin CAPture kit was also explored. Oligosaccharide–bacterial binding was then monitored using a Biacore X system.

## Materials and methods

### Materials

Mueller–Hinton broth, Brain Heart Infusion broth, and *Campylobacter* selective supplement were purchased from Oxoid (Basingstoke, Hampshire, UK). 2′-fucosyllactose was purchased from Carbosynth (Compton, Berkshire, UK). The Biacore X instrument, CM5 chip, SA chip, biotin CAPture kit, HBS–EP buffer (10 mM Hepes, 150 mM NaCl, 3.8 mM ethylenediaminetetraacetic acid [EDTA], and 0.05% [v/v] Tween 20), and amine coupling kit were purchased from GE Healthcare (Buckinghamshire, UK). The ImmunoPure goat anti-biotin antibody, EZ-Link biotin–$PEG_4$–hydrazide, sodium *meta*-periodate, 0.1 M sodium acetate buffer (pH 5.5), and Zeba desalt spin column were purchased from Pierce (Rockford, IL, USA).

### Bacterial strain and culture conditions

Bacterial strains used in this study are listed in Table 1. *C. jejuni* 81-176, a well-characterized invasive strain, was kindly donated by Marguerite Clyne (University College of Dublin, Ireland). The strain was cultured directly from storage onto Mueller–Hinton agar containing *Campylobacter* selective supplement and grown under micro-aerophilic conditions for 48 h at 37 °C. All other bacterial strains were grown aerobically for 12–24 h at 37 °C in their respective media (listed in Table 1). Bacterial cells were washed three times in HBS–EP buffer and resuspended to a concentration of $1 \times 10^8$ colony-forming units (CFU)/ml for screening studies unless otherwise stated.

**Table 1**
List of bacterial strains.

| Bacterial strain | Growth Media | Reference |
|---|---|---|
| *Pseudomonas aeruginosa* PAO1 | Luria–Bertani | Stover et al., [19] |
| *Cronobacter sakazakii* ATCC12868 | Luria–Bertani | ATCC |
| *Salmonella enterica* serotype Typhimurium DPC 6047 | Brain Heart Infusion | TFRC |
| *Staphylococcus aureu* subsp. *aureus* Rosenbach ATCC 29213 | Brain Heart Infusion | ATCC |
| *Campylobacter jejuni* 81-176 | Muller–Hinton | Byrne et al., [20] |
| *Listeria monocytogenes* DPC 6179 | Brain Heart Infusion | TFRC |
| *Streptococcus dysalactiae* DPC 5345 | Tryptic Soy | TFRC |
| Streptococcus mutans DPC 6143 | Tryptic Soy | TFRC |

*Note:* ATCC, American Type Culture Collection; TFRC, Teagasc Food Research Centre.

### Biotinylation of 2′-fucosyllactose

The biotinylation of 2′-fucosyllactose was performed using EZ-Link biotin–$PEG_4$–hydrazide as per the manufacturer's manual. Briefly, 1 ml of cold sodium *meta*-periodate solution (20 mM periodate) was added to 1 ml of 0.1 M sodium acetate buffer (pH 5.5) containing 0.5 mg of dissolved 2′-fucosyllactose, and the solution was mixed well. The mixture was then protected from light and incubated for 30 min at room temperature. Excess periodate was removed using a Zeba desalt spin column equilibrated with 0.1 M sodium acetate buffer (pH 5.5). Then 1 part of 50 mM biotin–hydrazide solution was added to 9 parts of the treated sample and incubated for 3–4 h at room temperature. The sample was then passed sequentially through two Zeba desalt spin columns to ensure that all unbound biotin was removed.

### Assessment of interaction of C. jejuni 81-176 with 2′-fucosyllactose using classical Biacore chip technology

All analyses were carried out on a Biacore X instrument at a constant temperature (25 °C) and flow rate (10 μl/min), unless otherwise stated, using HBS–EP as the run buffer.

#### CM5 chip

Initially, the CM5 chip was prepared for the capture of biotin-labeled 2′-fucosyllactose. Optimal immobilization conditions for the ImmunoPure goat anti-biotin antibody were established by diluting the antibody (50 μg/ml) in 10 mM sodium acetate buffer with varying pH values (4.0–5.5, increasing steps of 0.5). The various antibody solutions were injected over an inactive CM5 chip for a contact time of 2 min. The optimal immobilization solution was 10 mM sodium acetate (pH 4.5), whereas the optimal regeneration solution for the CM5 chip was glycine–HCl (pH 2.0), established as per the Biacore sensor surface handbook. The ImmunoPure goat anti-biotin antibody was immobilized to the CM5 chip through amine coupling. Briefly, the chip surface was activated by mixing 60 μl of 100 mM *N*-hydroxysuccinimide (NHS) with 60 μl of 400 mM *N*-ethyl-*N*-(dimethyl-aminopropyl)carbodiimide hydrochloride (EDC) and injecting 90 μl of the mixture over the chip surface. The antibody was then injected over the active chip surface, which consistently registered a signal response of 14,500–15,500 response units (RU). The theoretically calculated binding capacity of the chip surface ($R_{max}$), therefore, was determined to be between 190 and 210 RU. The $R_{max}$ was determined through the use of the following formula: $R_{max}$ = analyte/ligand (MW) × immobilized antibody (RU) × stoichiometric ratio.

Once the chip was prepared, it was then used to monitor whole *C. jejuni* 81-176 cell binding to 2′-fucosyllactose. Biotin-labeled 2′-fucosyllactose resuspended in HBS–EP buffer (50 μg/ml) was injected over the chip surface. *C. jejuni* 81-176 cells harvested from supplemented Mueller–Hinton agar plates, washed with HBS–EP running buffer, and adjusted to varying concentrations ($10^4$, $10^5$, $10^6$, $10^7$, $10^8$, and $10^9$ CFU/ml) were then passed over the surface separately for a contact time of 10 min. After each injection, the chip surface was regenerated with glycine–HCl (pH 2.0), and 2′-fucosyllactose was again immobilized to the chip surface prior to

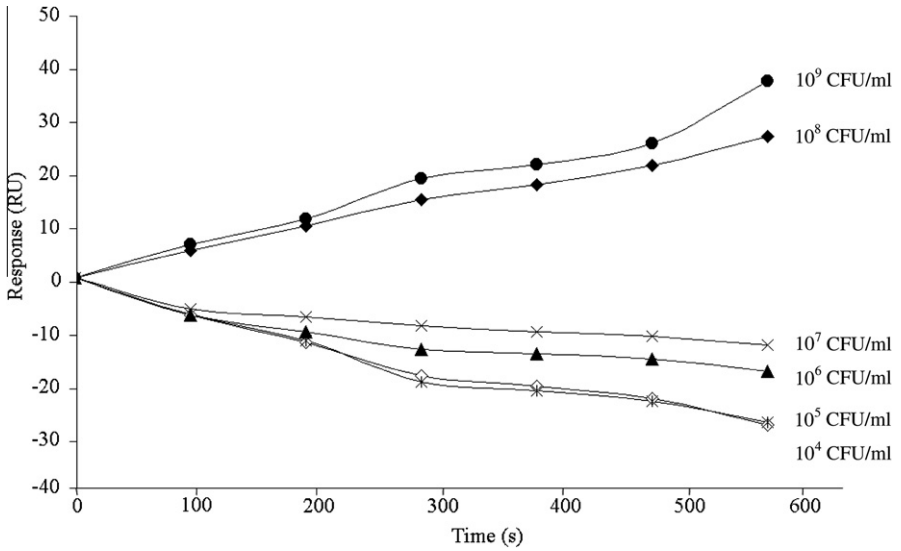


**Fig.1.** Concentration-dependent binding of *C. jejuni* 81-176–2′-fucosyllactose immobilized on the CM5 chip surface. Bacterial concentrations were $10^9$ CFU/ml (●), $10^8$ CFU/ml (♦), $10^7$ CFU/ml (×), $10^6$ CFU/ml (▲), $10^5$ CFU/ml (ж), and $10^4$ CFU/ml (◇).

the introduction of the next bacterial concentration. Once the baseline had stabilized post-injection, the increase in the signal response was recorded in triplicate over time.

*SA chip*

The streptavidin-coated chip surface was prepared for optimal performance by a short pulse injection of 1 M NaCl and 50 mM NaOH (filtered and degassed). Biotin-labeled 2′-fucosyllactose was diluted in HBS–EP buffer (50 μg/ml), and 100 μl of this solution was injected over the surface at a flow rate of 10 μl/min. The chip surface was then washed with HBS–EP buffer to ensure the removal of any non-immobilized oligosaccharides. Subsequently, whole *C. jejuni* 81-176 cells were harvested from supplemented Mueller–Hinton agar plates, washed, and resuspended in HBS–EP running buffer. The cell suspension ($1 \times 10^8$ CFU/ml) was then injected over the chip surface at two different flow rates, 5 and 10 μl/min, and the binding signal was then measured.

*Validation of C. jejuni 81-176 binding to 2′-fucosyllactose*

To validate our techniques, *C. jejuni* 81-176 was incubated with unlabeled 2′-fucosyllactose prior to injecting the bacteria over the biotin-labeled 2′-fucosyllactose immobilized to the Biacore chip surface. Briefly, *C. jejuni* 81-176 ($1 \times 10^8$ CFU/ml) was harvested from supplemented Mueller–Hinton agar plates and resuspended in running buffer containing unlabeled 2′-fucosyllactose (50 μg/ml). The cell suspension was then incubated at 37 °C for 30–60 min. After incubation, 100 μl of the mixture was injected over the immobilized biotin-labeled 2′-fucosyllactose on the chip at a flow rate of 10 μl/min. The binding signal was measured in triplicate over time.

*Screening pathogens for the ability to bind 2′-fucosyllactose using the Biacore biotin CAPture kit*

The sensor chip CAP was rehydrated prior to analysis by passing HBS–EP buffer over its surface for 24–48 h. Interaction analysis was performed as per the manufacturer's manual. Briefly, the biotin CAPture reagent was injected over the chip surface at a reduced flow rate of 2 μl/min for 5 min. Subsequently, 100 μl injections of biotin-labeled 2′-fucosyllactose were passed over the surface, followed by *C. jejuni* 81-176 ($1 \times 10^8$ CFU/ml), at a flow rate of 10 μl/min. The chip surface was regenerated by a short 2 min injection of guanidine–HCl and NaOH. This process was repeated for each of the following bacteria in triplicate: *Listeria monocytogenes, Salmonella enteria* serovar Typhimurium, *Staphylococcus aureus, Cronobacter sakazakii, Streptococcus mutans, Streptococcus agalactiae,* and *Pseudomonas aeruginosa.*

*Statistical analysis*

All experiments were performed on three separate occasions in triplicate. Results are presented as the means ± standard deviations of replicate experiments. Nonspecific binding of the analyte to the test surface was eliminated from all experiments through the use of a reference surface.

## Results and discussion

In the current study, the applicability of using SPR to detect the interaction between the HMO, 2′-fucosyllactose and whole *C. jejuni* cells was assessed in "real time." In addition, the technology was exploited to detect potential interactions between this milk oligosaccharide and other pathogenic bacteria. The CM5 chip proved to be successful in monitoring the binding of *C. jejuni* 81-176 to 2′-fucosyllactose in a concentration-dependent manner. The theoretical binding capacity was determined to range between 190 and 210 RU. Considering this, the chip surface was deemed to be fully saturated after the 2′-fucosyllactose injection given that a response of 190 ± 1.56 RU was observed. Thereafter, injections of $10^8$ and $10^9$ CFU/ml *C. jejuni* resulted in signal responses of 15 ± 0.58 and 31 ± 1.25 RU, respectively (Fig. 1). This increase was fully attributed to bacterial binding because any nonspecific binding to the chip surface was eliminated through the use of a reference surface. The detection of bacterial binding was limited to samples with concentrations of organisms greater than $1 \times 10^8$ CFU/ml. Indeed, the detection of whole cell binding in such studies is often associated with samples containing at least $10^5$ whole cells [14–16]. This is not a reflection on the ability of the bacterial cell to bind the immobilized structure; rather, it is the concentration of bacteria that is required to generate a measurable signal. The sensor chip surface consists of a dextran matrix, with the immobilized ligand scaling the length of each dextran strand. The field of detection of the chip surface is limited to approximately 300 nm. Therefore,

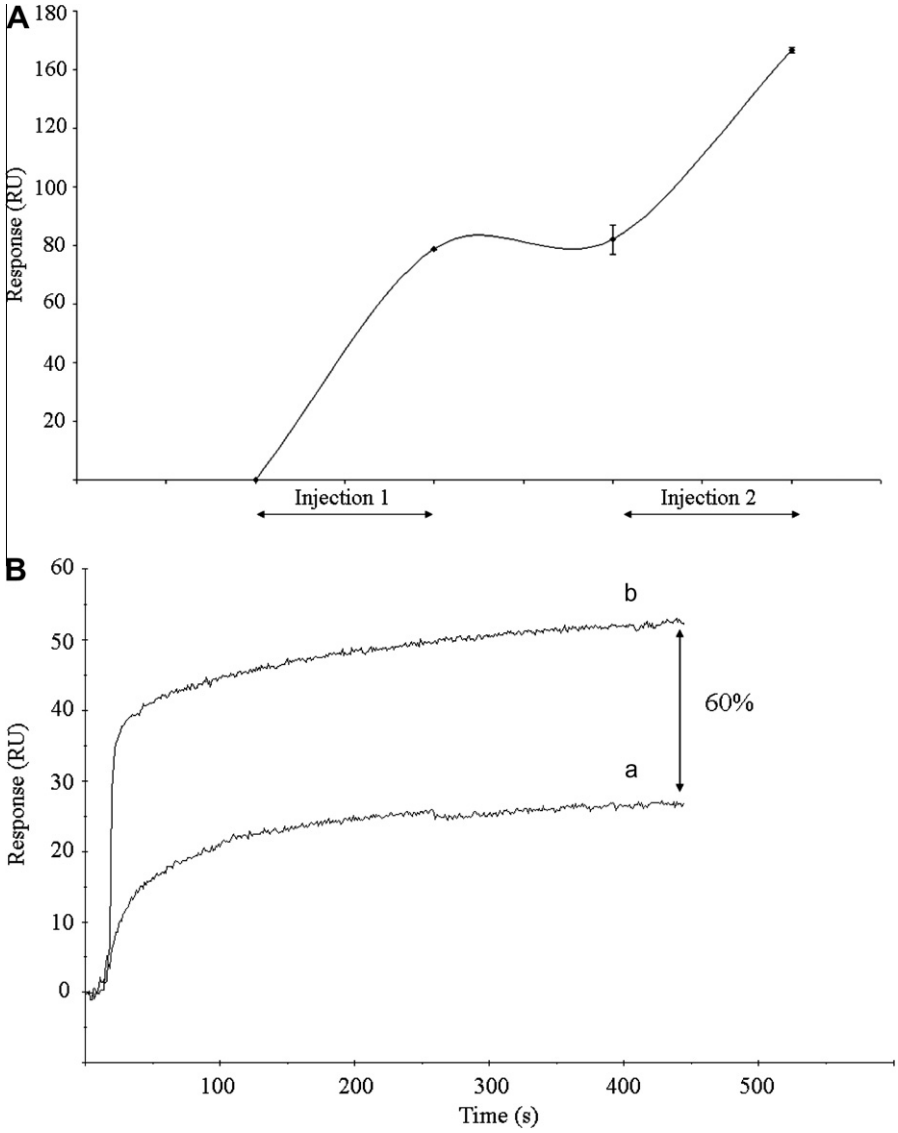


**Fig.2.** Binding interactions using a streptavidin-coated dextran surface. (A) An SA Biacore chip was used to monitor the binding of *C. jejuni* to 2′-fucosyllactose. Biotin-labeled 2′-fucosyllactose immobilized to the SA chip (injection 1) was bound by whole *C. jejuni* cells (injection 2). (B) A comparative study to validate the use of the SA chip to monitor the *C. jejuni*–2′-fucosyllactose interaction is shown. Unlabeled 2′-fucosyllactose was used to block binding of *C. jejuni* to biotin-labeled 2′-fucosyllactose (curve a). The control was untreated (curve b).

large molecules such as whole bacterial cells, which often fail to penetrate the dextran matrix, bind beyond the limits of detection and, thus, register a low signal response.

To increase the sensitivity and specificity of the measurement area, a streptavidin-coated (SA) chip surface, prepared for the high-affinity capture of biotinylated ligands, was used and thus eliminated the use of the anti-biotin antibody and the CM5 chip. This would enable the streptavidin–biotin–2′-fucosyllactose complex, in the absence of a capturing antibody, to lie closer to the chip surface, resulting in the generation of an increased signal response. Fig. 2A illustrates the success of this technique, with this interaction registering a higher signal response than that of the CM5 chip. Indeed, 2′-fucosyllactose immobilization resulted in an average baseline shift of 82 ± 5.03 RU, which then increased to 147 ± 0.57 RU due to the introduction of $1 \times 10^8$ CFU/ml whole *C. jejuni* 81-176 cells at 5 μl/min. Alternatively, a flow rate of 10 μl/min resulted in a bacterial–2′-fucosyllactose interaction that registered an increased signal response, illustrating that this interaction was flow-rate dependent. These results demonstrate the importance of stringent optimization when using all Biacore biosensor techniques. Bacterial binding to surface structures is often limited by the fact that the bacterial cell must overcome hydrodynamic resistance forces. However, in this study a high-affinity interaction was observed given that the flow of bacteria was relatively high with a large contact time (10 min). The reduced response observed at the lower flow rate of 5 μl/min was most likely a consequence of "stack interference" or the "mass transit effect" [11]. This occurs when bacteria "stack" by forming a small barrier at the entrance of the active flow cell, limiting the access of the bacteria to all attachment sites available. Higher flow rates reduce the possibility of this occurring by spreading the bacteria over a large surface area and, therefore, registering an increased response.

To substantiate the SPR method as a way of detecting the interaction between the bacteria and the HMO, *C. jejuni* 81-176 was preincubated with unlabeled 2′-fucosyllactose. In theory, the unlabeled sugar should occupy the binding sites of a subpopulation of bacteria, resulting in a reduced response once passed over the pre-immobilized chip surface. Indeed, a 60% reduction in the signal response was observed when *C. jejuni* 81-176 was preincubated with unlabeled 2′-fucosyllactose, demonstrating that many of the

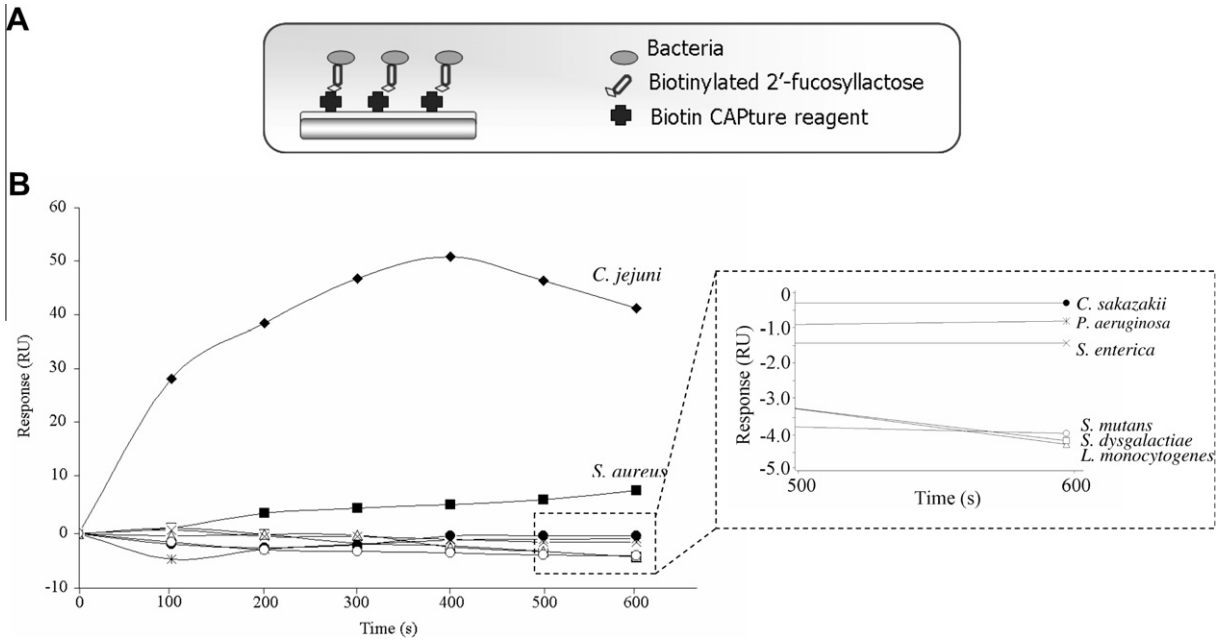


**Fig.3.** Use of the Biacore biotin CAPture kit to monitor the binding of a range of pathogenic bacteria to 2′-fucosyllactose. (A) Illustration of the biotin CAPture test surface used to monitor whole bacterial cell binding. (B) Sensorgram demonstrating the binding of 2′-fucosyllactose to the following pathogens: *Campylobacter jejuni* (♦), *Staphylococcus aureus* (■), *Cronobacter sakazakii* (●), *Pseudomonas aeruginosa* (⁎), *Salmonella enterica* (×), *Streptococcus mutans* (○), *Streptococcus dysgalactiae* (□), and *Listeria monocytogenes* (Δ).

potential bacterial receptors for the immobilized biotin–2′-fucosyllactose were now occupied by its unlabeled equivalent (Fig. 2B). Unbound unlabeled 2′-fucosyllactose did not contribute to the signal response generated given that injections of this compound alone failed to register a response. Blocking binding sites in this manner represents an attractive approach to validating bacterial binding to immobilized oligosaccharides.

To determine whether SPR could be used to screen a variety of pathogens for interactions with 2′-fucosyllactose, the biotin CAPture kit was employed. The newly developed biotin CAPture kit involves the use of a carboxymethylated dextran surface to which single-stranded DNA (ssDNA) has been pre-immobilized. By introducing a complementary strand of ssDNA conjugated with streptavidin, a platform is created for biotin capture (Fig. 3A). This surface can then be regenerated by breaking the interaction between the two complementary strands of DNA. The interaction between streptavidin and biotin is often irreversible, and any attempt to disrupt this interaction often destroys the binding capacity of the chip surface. Therefore, the biotin CAPture kit represents a unique approach to alleviate this problem because each kit is capable of withstanding 80–140 assay cycles. During this screen, the *C. jejuni* 81-176–2′-fucosyllactose interaction was used as a positive control. *P. aeruginosa* PAO-1 was used as a negative control because a previous study by Perret and coworkers [21] demonstrated that 2′-fucosyllactose was a poor inhibitor of the dominant bacterial lectin PA-IIL of *P. aeruginosa*. Indeed, the interaction between *C. jejuni* 81-176 and 2′-fucosyllactose yielded a signal response of 39.03 ± 7.79 RU, whereas *P. aeruginosa* PAO-1 failed to generate a response with the oligosaccharide (Fig. 3B). All other pathogens screened failed to register a signal response with the exception of *S. aureus,* which had a consistent baseline increase of 7.76 ± 0.90 RU. Interestingly, there are no data available on *S. aureus* binding HMOs; therefore, the use of SPR may represent a way of detecting novel interactions between bacterial strains of this organism and milk oligosaccharides. The results derived from the biotin CAPture kit proved to be highly reproducible. In 24 cycles of ligand immobilization and regeneration, the performance of the chip did not deteriorate. The immobilization of the biotin CAPture reagent was consistent for each injection (4002.62 ± 3.21 RU), and the capture of biotin-labeled 2′-fucosyllactose averaged an increase of 26.34 ± 1.43 RU above the baseline. Nonspecific binding of the bacteria to the chip surface, which proved to be strain dependent, was easily eliminated through the use of a reference surface. This study has presented a highly reproducible, cost-effective, sensitive, and controlled high-throughput screen for milk oligosaccharide binding to whole bacterial cells.

The use of SPR in this study has offered a fast and simple method to monitor the dynamic interactions between whole bacterial cells and biotin-labeled milk oligosaccharides. Through three separate assays, the binding of *C. jejuni* 81-176–2′-fucosyllactose was clearly demonstrated, with the generation of a signal response being directly related to the levels of bacterial exposure. It is not surprising that no signal response was generated at the lower levels of bacterial exposure given that for relatively large molecules, such as bacterial cells, a response is dependent on the cell geometry and distribution over the detector surface. As a consequence, it is not possible to directly translate a response in RU to a corresponding number of cells bound. The use of SPR for biological binding studies will always have its limitations, but new advances make this technology a great tool in gaining an understanding of the dynamic biological interactions that drive and regulate biological processes such as bacterial binding within the gastrointestinal tract.

## Concluding remarks

The methods outlined in this study have potential as alternative prescreens in studies aiming to identify anti-infective oligosaccharides. Exploiting such approaches may be preferable to using cell line models because biosensor-based assays can be completed in hours rather than weeks. Indeed, the detection of oligosaccharide–bacterial binding was determined by all three techniques in less than 40 min, demonstrating the high-throughput manner of such technology. Indeed, it is tempting to suggest that the

approaches developed in this study may be applied to detect oligosaccharide interactions with any ligand or binding partner of choice.

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