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RACHEL MENEZES CASTELO

DESENVOLVIMENTO DE IMUNOSSENSOR ELETROQUÍMICO COM O USO DE PONTO QUÂNTICO DE AMÊNDOA DE PEQUI PARA DETECÇÃO DE *Salmonella* Sp

FORTALEZA-CEARÁ

2024

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Tese apresentada ao Curso de Doutorado Acadêmico em Ciências Naturais, do Programa de Pós-Graduação em Ciências Naturais, Centro de Ciências e Tecnologia da Universidade Estadual do Ceará, como requisito parcial à obtenção do título de Doutor em Ciências Naturais. Área de concentração: Aproveitamento de Recursos Naturais.

Orientador: Prof. Dr. Carlucio Roberto Alves

Coorientadora: Profa. Dra. Roselayne Ferro Furtado

Coorientador: Prof. Dr. Sérgio Antônio Spinola Machado

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Prof. Dr. Carlucio Roberto Alves

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TEREZINHA FEITOSA MACHADO
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LUANA GUABIRABA MENDES
Data: 02/12/2024 09:29:08-0300
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Profa. Dra. Luana Guabiraba Mendes
(Examinadora)

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"Um cientista no seu laboratório não é apenas um técnico: é, também, uma criança colocada à frente de fenômenos naturais que impressionam como se fossem um conto de fadas."

(Marie Curie)

RESUMO

A frequência e a gravidade das doenças causadas pelo consumo de água e alimentos contaminados com *Salmonella* incentivam o desenvolvimento de métodos rápidos e sensíveis para detecção dessa bactéria. Portanto, a detecção rápida e específica de *Salmonella* em alimentos é essencial para garantir a segurança dos alimentos. A utilização de pontos quânticos (de carbono, Cdots) em biossensores têm avançado significativamente, acrescentando melhorias a diferentes técnicas de detecção, podendo estes substituirem enzimas e outras moléculas no uso como marcadores. Neste estudo, um imunossensor amperométrico para *Salmonella* Thyphimurium foi desenvolvido utilizando anticorpos marcados com pontos quânticos de carbono (Cdots) de amêndoa de pequi (*Caryocar coriaceum*). Os Cdots foram sintetizados por pirólise e caracterizados por Espectroscopia no Infravermelho por Transformada de Fourier (FTIR), espectroscopia no ultravioleta visível (UV/fluorescência), eletroquímica, potencial zeta e microscopia eletrônica de transmissão (TEM). Foi estimado um tamanho de partícula de 6,80 ± 2,13 nm e o potencial zeta foi de -47,4 mV, indicando a presença, preponderante, de grupos ácidos. A avaliação da impedância da resposta dos biossensores montados para *Salmonella* ativa (Rct = 13,4 kΩ) e inativa (Rct = 499,7 Ω) mostrou diferença significativa em seus valores, concordância com os obtidos por análises cronoamperométricas, que tiveram seus valores de corrente drasticamente reduzidos de -2,2 mA (ativa) a 0 mA (inativa). Foi estabelecida uma curva analítica para *Salmonella* com limite de detecção (LOD) inferior a 1 UFC/mL. O imunossensor marcado com Cdots da amêndoa do pequi demonstrou especificidade frente a outros microrganismos e foi eficaz na detecção de *Salmonella* em alimentos como queijo e em água residual de alimentos (frangos e ovos), permanecendo com LOD muito baixo, abaixo de 1 UFC/mL. A conjugação de Cdots ao anticorpo secundário utilizado na identificação da *Salmonella* foi determinante para o sucesso do dispositivo. Este estudo destaca o uso potencial do biossensor amperométrico com anticorpo secundário conjugado a pontos de carbono de Pequi para uma detecção rápida e sensível de *Salmonella* em amostras de alimentos.

Palavras-chave: Pontos de carbono; *Caryocar coriaceum*; Imunossensor; Amêndoa de Pequi; *Salmonella*.

ABSTRACT

The frequency and severity of outbreaks caused by *Salmonella* in food and water encourage the advancement of rapid and sensitive methods for detecting microorganisms to protect public health. Therefore, fast and specific detection of *Salmonella* in food is essential to ensure food safety. The use of quantum dots (carbon, Cdots) in biosensors has advanced significantly, adding improvements to different detection techniques, and they can replace enzymes and other molecules used as markers. This study designed an amperometric immunosensor for *Salmonella* Thyphimurium using antibodies labeled with carbon quantum dots (Cdots) from pequi almond (*Caryocar coriaceum*). The Cdots were synthesized by pyrolysis and characterized by FTIR, UV/fluorescence, electrochemistry, zeta potential, and transmission electron microscopy (TEM). The particle size was estimated at 6.80 ± 2.13 nm, and the zeta potential was -47.4 mV, indicating the predominant presence of acidic groups, confirmed by FTIR. The evaluation of the response impedance of the biosensors assembled for live (Rct = 13.4 kΩ) and dead (Rct = 499.7 Ω) *Salmonella* were in agreement with the results obtained by chronoamperometric analyses, which had their current values drastically reduced from -2.2 mA (live) to 0 mA (dead). An analytical curve for *Salmonella* was established with a limit of detection (LOD) lower than 1 CFU/mL. The immunosensor labeled with Cdots from the pequi almond demonstrated excellent specificity against other microorganisms and effectively detected *Salmonella* in foods such as cheese and waste water of food as eggs and chicken, remaining with a very low LOD, below 1 CFU/mL. The bioconjugation between Cdots and the secondary antibody used in identifying *Salmonella* was decisive for the device's success. This study highlights the potential use of the amperometric biosensor with secondary antibody conjugated with pequi carbon dot for rapid and sensitive detection of *Salmonella* in various samples.

Keywords: Carbon dots; *Caryocar brasiliensis* Cambess; Immunosensor; Pequi-nut; *Salmonella*.

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1 INTRODUÇÃO

As infecções por *Salmonella* causam várias morbidades e mortalidade em todo o mundo, especialmente em países em desenvolvimento (KURTZ; GOGGINS; MCLACHLAN, 2017), sendo um dos surtos de doenças transmitidas por alimentos mais prevalentes, principalmente em países em desenvolvimento, incluindo África, Ásia e América Latina (BROCKETT *et al*., 2020; CRUMP, 2019; OLOBATOKE, 2017; PHOON; CHAN; KOH, 2015). A prevalência é alta em regiões de poucos recursos onde o saneamento é precário e a falta de abastecimento de água potável é um problema (WADDINGTON; DARTON; POLLARD, 2014). Garantir que a água e alimentos saudáveis sejam acessíveis a todos tem sido um desafio global.

As técnicas convencionais, baseadas em métodos clássicos de culturas de bactérias, são consideradas sensíveis e confiáveis, no entanto, dependem de uma sequência complexa de etapas e requerem vários dias para a obtenção do resultado (AWANG *et al*., 2021), demandando um tempo maior para um resultado efetivo e possíveis medidas para evitar um alto índice de contaminação. O uso de testes rápidos e biossensores são opções para se conseguir resultados mais rápidos e sensíveis.

A aplicação de estratégias baseadas em biossensores para detectar microorganismos tornou-se um campo de pesquisa promissor (AWANG *et al*., 2021; RAMÍREZ-CASTILLO *et al*., 2015; RENGARAJ *et al*., 2018). A utilização de biossensores forneceu identificação em tempo real da contaminação microbiológica, enquanto as técnicas tradicionais levam vários dias para obter o resultado (EJEIAN *et al*., 2018; YAROSHENKO *et al*., 2020). Recentemente, muitos dispositivos portáteis são projetados para monitorar amperometricamente vários microrganismos por seus fenômenos de eletro-oxidação ou eletrorredução (AWANG *et al*., 2021; KOKKINOS; ECONOMOU, 2017; SOUSA *et al*., 2018).

Os biossensores oferecem muitos benefícios sobre os métodos baseados em laboratório, incluindo custo-benefício, portabilidade, prováveis aplicações *in situ*, alta precisão, sensibilidade, especificidade e superioridade na resposta rápida (ROTARIU *et al*., 2016). Nanomateriais de carbono têm sido aplicados, desde a simples transdução de sinal até o reconhecimento biológico da interação molecular entre receptores e analitos alvo em plataformas de biossensores. Devido à estabilidade química, condutividade elétrica robusta, alta área superficial, biocompatibilidade durável e alta resistência mecânica, a aplicação de

nanomateriais de carbono tem crescido de forma eficiente na área de tecnologia de biossensores (ABEDI *et al*., 2021; BIJU, 2014; MOMBINI *et al*., 2019).

Os Pontos Quânticos (PQ) são um novo membro da família de nanomateriais à base de carbono de dimensão zero com diâmetros inferiores a 10 nm, conhecidos por suas propriedades exclusivas de fluorescência. PQs foram descobertos por acaso por Xu *et al*. ao purificar nanotubos de carbono de parede simples (SWCNTs) por eletroforese(XU *et al*., 2004). Nanomateriais de carbono têm sido aplicados desde a simples transdução de sinal até o reconhecimento biológico da interação molecular entre receptores e analitos alvo em plataformas de biossensores. Pesquisas com PQ aplicados em biossensores eletroquímicos, surgiram recentemente, a atuação dessas nanopartículas nessas plataformas não está bem definida. Vários fatores podem influenciar dispositivos mais efetivos, tais como: tipo de semicondutor que compõe a nanopartícula, tipo de eletrodo utilizado, tipo de agente estabilizante/funcionalizante empregado na síntese dos PQs, modificações realizadas no eletrodo e até mesmo o tamanho da nanopartícula (JI *et al*., 2020).

O uso de resíduos vegetais como fonte de síntese de PQs tem ganhado destaque, tendo como principais vantagens contribuir para reaproveitamento de subprodutos vegetais para produção de materiais com alto valor agregado (FAN et al., 2020; BAWEJA, JEET, 2019). O pequi (*Caryocar coriaceum*) é uma espécie arbórea nativa do cerrado brasileiro muito utilizada para alimentação devido suas características nutricionais. A amêndoa do pequi se destaca por suas características físico-químicas, apresentando um alto teor de lipídeos, carboidratos e proteínas (OLIVEIRA *et al*., 2010), podendo representar uma fonte promissora para síntese de novos materiais (DE OLIVEIRA; DA SILVA ABREU, 2021) e desta forma o aproveitamento da amêndoa representaria mais uma fonte de renda para economia local.

O presente estudo teve o objetivo de desenvolver, caracterizar e avaliar o desempenho analítico de um imunosensor eletroquímico utilizando *carbon dots* de pequi como marcador de anticorpo secundário na resposta analítica para a identificação e quantificação de *Salmonella* sp.

2 REFERENCIAL TEÓRICO

2.1 Doenças transmitidas por alimentos (DTA)

A DTA é causada pela ingestão de alimentos ou água contaminada por substâncias químicas ou microrganismos, tais como as bactérias, vírus, parasitas ou por toxinas produzidas por estes contaminantes, com intoxicações e infecções que podem ser agudas (causando sintomas imediatos) e crônicas. No geral, os sintomas causados são a diarreia (que muitas vezes sanguinolenta), náuseas, vômitos e dores abdominais, que são características de infecção alimentar e também por manchas e prurido pelo corpo, que são característicos de intoxicação (CDC, 2022; WHO, 2018).

Food and Drug Administration (FDA) e Centers for Disease Control and Prevention (CDC) são autoridades internacionalmente reconhecidas no que diz respeito à segurança de alimentos. Nos EUA, o FDA avalia o potencial de exposição e riscos e fornece informações sobre a prevenção de doenças transmitidas por alimentos. O CDC, especificamente, é responsável pelo monitoramento dos surtos e casos de enfermidades de origem alimentar nos Estados Unidos, e estima que a *Salmonella* cause cerca de 1,2 milhão de doenças, 23.000 hospitalizações e 450 mortes a cada ano. Os departamentos de saúde pública identificam e investigam surtos de doenças de origem alimentar causadas por agentes entéricos bacterianos, virais, parasitárias e químicos / tóxicos e apontam os alimentos como a origem de cerca de 1 milhão dessas enfermidades (CDC, 2022)

A Organização Mundial de Saúde (*World Health Organization* - WHO) estima que doenças diarreicas transmitidas por água e por alimentos contaminados sejam responsáveis por cerca de 2,2 milhões de óbitos anualmente, sendo 1,9 milhão de crianças (WHO, 2018). Considerada uma das principais causas de doenças transmitidas por alimentos em todo o mundo, as bactérias do gênero *Salmonella* são geralmente transmitidas aos seres humanos através do consumo de alimentos contaminados e água (CDC, 2022; CRUMP, 2019; FERREIRA, 2021; SANTOS *et al*., 2020).

2.2 *Salmonella* spp.

O gênero *Salmonella,* pertencente à família Enterobacteriaceae e a tribo Salmonellae, apresenta forma de bastonetes, Gram-negativos, não formadores de esporos. O gênero *Salmonella* é dividido em duas espécies que podem causar doenças em humanos: *Salmonella enterica* e *Salmonella* bongori (HAMMACK, 2012). A espécie *S. enterica* é a que representa maior risco à saúde pública, estando associada à maioria dos casos de doenças

transmitidas por alimentos (DTAs). Para propósitos epidemiológicos, os sorotipos de *Salmonella* podem ser divididos em três grupos:

a) sorotipos que infectam somente o homem – *Salmonella enterica* subespécie *enterica* sorovar Typhi (*S.* Typhi) e *S*. Parathyphi, causadores da febre tifoide e paratifoide, respectivamente;

b) sorotipos adaptados a hospedeiros específicos – alguns são patógenos humanos;

c) sorotipos não-adaptados (sem preferência por hospedeiro) – patogênicos ao homem e outros animais.

Salmonella enterica subespécie *enterica* sorovar Typhi (*Salmonella* Typhi) é a causa da febre tifóide. Juntos, *Salmonella* Typhi e *Salmonella* sorovar Paratyphi A são os principais agentes da febre entérica. Como outros sorovares de *Salmonella* tifóide, *Salmonella* Typhi é um organismo restrito ao hospedeiro humano. O papel da água como veículo para a febre tifóide tem sido apreciado desde o final de 1800 (KURTZ; GOGGINS; MCLACHLAN, 2017; WADDINGTON; DARTON; POLLARD, 2014) e o papel dos alimentos não muito tempo depois (GORDON *et al*., 2020).

Além da divisão em espécies e subespécies o gênero *Salmonella* é comumente subdividido em sorotipos baseado no esquema de Kaufmann-White, publicado pela primeira vez em 1934, que diferencia as estirpes de *Salmonella* por suas propriedades antigênicas superficiais e flagelares. Em cada subespécie são reconhecidos diferentes números de sorovares tendo por base a caracterização de seus antígenos somáticos (O), flagelares (H) e capsulares (Vi) (SILVA; JUNQUEIRA; SILVEIRA, 2021).

De acordo com o Centro de Referência e Pesquisa em *Salmonella* do Instituto Pasteur (França), colaborador da WHO, existem, 2.579 sorotipos, destes 2.557 pertencem à espécie *S. enterica* e 1.531 à subespécie enterica. Os sorogrupos somáticos (O) mais comuns são A, B, C1, C2, D, E1 e E4 e correspondem a aproximadamente 99% das infecções por *Salmonella* causadas em humanos e animais de sangue quente (WHO, 2018). No Brasil, a Agência Nacional de Vigilância Sanitária (Anvisa), através da Instrução Normativa nº 161 de 1 de julho de 2022, que estabelece padrões microbiológicos para alimentos, também determina que o alimento próprio para o consumo humano deve apresentar ausência de *Salmonella* spp., em 25 g ou mL (ANVISA, 2022). Desta forma, testes que apresentem um alto padrão de confiabilidade e sejam rápidos em suas respostas são necessários.

Bactérias do gênero *Salmonella* podem causar dois tipos de doenças, de acordo com o sorotipo: a salmonelose e a febre tifoide (além da paratifoide, uma forma mais branda que a anterior). A sintomatologia da salmonelose é caracterizada por náuseas, vômitos, dores abdominais, diarreia, febre e dores de cabeça, podendo durar de dois a sete dias. Apresenta uma baixa taxa de mortalidade, e em organismos jovens e saudáveis é considerada como uma doença autolimitada. O mecanismo de ação da bactéria permite que ela penetre na mucosa intestinal promovendo sua multiplicação. Em alguns casos, o patógeno consegue atravessar a mucosa intestinal e invade os sistemas linfático e cardiovascular, podendo se disseminar e, eventualmente, afetar outros órgãos. A febre associada às infecções por *Salmonella* ocorre devido à liberação de endotoxinas, quando as células bacterianas são lisadas (HAMMACK, 2012; SILVA; JUNQUEIRA; SILVEIRA, 2021).

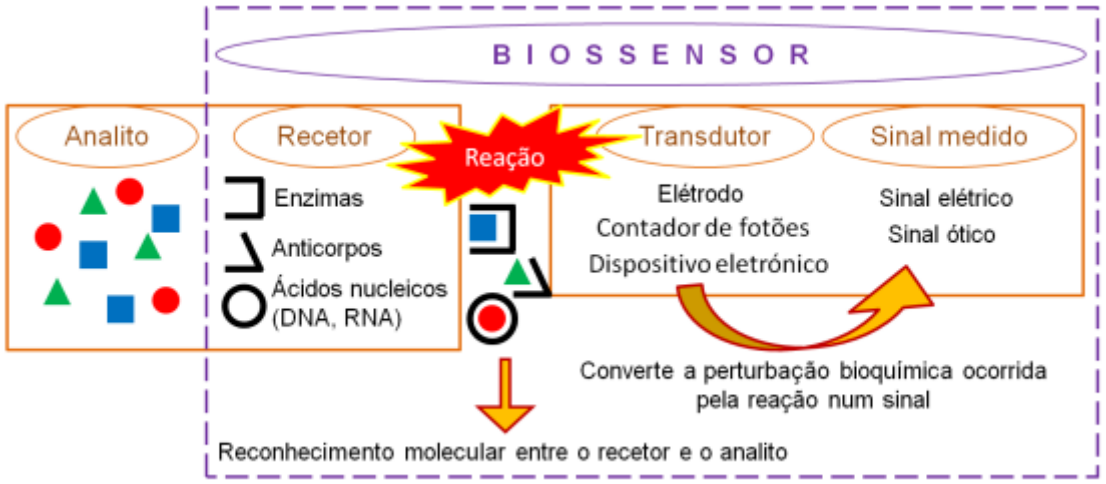
2.3 Biossensores

De acordo com a definição da *International Union of Pure and Applied Chemistry* (IUPAC, 2014), um biossensor é um dispositivo que utiliza reações bioquímicas específicas mediadas por enzimas, imunosistemas, organelas, tecidos ou células para detectar compostos químicos, geralmente, por meio de sinais elétricos, térmicos ou ópticos. A interação do biossensor com o analito resulta na alteração de uma ou mais propriedades físico-químicas (modificação de pH, transferência de elétrons, variação de massa, transferência de calor, liberação de gases ou íons) que são detectadas e mensuradas pelo transdutor. O principal objetivo é produzir um sinal eletrônico proporcional em magnitude e/ou frequência à concentração de um determinado analito ou grupo de analitos que interagem com o elemento biorreceptor. A parte eletrônica é constituída de um amplificador dos sinais elétricos e de um sistema de processamento dos dados (FURTADO *et al*., 2008).

De forma mais prática, pode-se dizer, que um biossensor é uma ferramenta analítica, constituída pelo biorreceptor, camada sensora do transdutor que reconhece o analito, e pelo transdutor que converte o evento de reconhecimento em sinal elétrico mensurável. O biorreceptor pode ser um tecido, microrganismo, organela, célula, enzima, anticorpo ou ácido nucleico. O transdutor pode ser óptico, eletroquímico, termométrico, piezoelétrico, magnético e micromecânico, ou combinações destes (VELUSAMY *et al*., 2010). As técnicas de investigação eletroquímicas variam bastante (voltametria linear, cíclica, e de pulso diferencial, espectroscopia de impedância eletroquímica, etc). De modo geral, é aplicado um potencial ao

sistema estudado e monitorada a corrente, ou do contrário, realizar um procedimento inverso (aplicar a corrente e monitorar o potencial) (RIBEIRO *et al*., 2017).

Figura 1. Esquema geral do funcionamento de um biossensor



Fonte: Justino; Rocha-Santos; Duarte (2013)

As medições experimentais são realizadas em uma célula eletroquímica contendo dois ou três eletrodos: (a) trabalho (WE), onde ocorrem os processos eletroquímicos de interesse; (b) referência (RE), que permite a aplicação de um potencial conhecido em relação ao eletrodo de trabalho e (c) auxiliar ou contraeletrodo (CE), utilizado para fluir a corrente do sistema, e em geral ocorrem processos eletroquímicos complementares para garantir a eletroneutralidade do sistema (RIBEIRO *et al*., 2017).

A demanda do mercado por técnicas rápidas e portáteis está impulsionando o desenvolvimento de biossensores. Repostas rápidas, específicas e de alta sensibilidade são fundamentais para o monitoramento preventivo de patógenos na cadeia produtiva das indústrias de alimentos. Os investimentos realizados para o desenvolvimento de biossensores aplicáveis nesta área convergem para a necessidade urgente de implantação de medidas que garantam a produção de alimentos seguros. A aplicação destes dispositivos reflete em ganhos à saúde pública e à economia dos países, por meio de monitoramento de DTAs e dos custos associados aos seus tratamentos (CINTI *et al*., 2017; MELO *et al*., 2016).

2.3.1 Imunossensores eletroquímicos

Os imunossensores são biossensores que utilizam como biorreceptor moléculas de anticorpo, e são comumente classificados pelo tipo de transdutor utilizado. Uma grande

variedade de transdutores foi desenvolvida recentemente para a detecção de patógenos transmitidos por alimentos. Estes se baseiam em métodos ópticos, onda acústica (piezoelétricos) e eletroquímicos (VELUSAMY *et al*., 2010).

Os imunossensores eletroquímicos do tipo amperométricos têm sido preferidos pela fácil manipulação e por trabalharem com potencial específico, reduzindo enormemente a interferência de espécies químicas. A concentração do analito é diretamente proporcional à alteração na corrente elétrica. Quando são utilizados como biorreceptores anticorpos ou DNA, a interação com o analito não gera íons redox, sendo necessária a conjugação a uma enzima como, por exemplo, a peroxidase que é usada para gerar e amplificar a resposta eletroquímica (PIMENTA-MARTINS *et al*., 2012). Uma grande variedade de imunossensores amperométricos para detecção de patógenos pode ser encontrada na literatura (DE BRITO *et al*., 2022; EJEIAN *et al*., 2018; MELO *et al*., 2016; SINGH *et al*., 2005). Uma etapa importante no desenvolvimento de um imunossensor eletroquímico que usa marcação é a escolha do marcador do anticorpo secundário e do procedimento utilizado para a obtenção da resposta analítica(RICCI; ADORNETTO; PALLESCHI, 2012).

Em comparação com os métodos tradicionais, a combinação de nanotecnologia e biossensores têm apresentado grandes vantagens: triagem de alto rendimento, limite de detecção aprimorado, análise em tempo real e tamanhos de amostra significativamente reduzidos (CHAUHAN; MAEKAWA; KUMAR, 2017; ZHANG *et al*., 2018).

2.4 Nanotecnologia e Biossensores

A constante busca por dispositivos cada vez menores e com características específicas, tem sido um dos principais objetivos nas áreas tecnológicas e científicas do século XXI. Dessa forma, a nanotecnologia tem se tornado um dos campos mais importantes e pode ser definida como a ciência envolvida na concepção, síntese, caracterização e aplicação de materiais em escala de dimensões físicas nanométricas (MARTINS; TRINDADE, 2012; WANG; HU, 2009).

Quando os materiais têm pelo menos uma de suas dimensões físicas de 1 a 100 nm são chamados de nanomateriais. Um material com apenas uma dimensão na escala nanométrica é chamado nanofilme, com duas dimensões é denominado nanofio (por exemplo, os nanotubos de carbono) e com três dimensões é chamado nanopartícula (por exemplo: fulerenos, dendrímeros ou pontos quânticos) (FARIA-TISCHER; TISCHER, 2012). As características destes nanomateriais, tais como, propriedades semicondutoras/condutoras ou

sua alta resistência à tensão física, entre outras, vêm sendo exploradas para o aperfeiçoamento de biossensores (RIBEIRO *et al*., 2017).

Nanomateriais com excelentes propriedades ópticas e eletroquímicas são extremamente bem-sucedidos e têm sido foco de pesquisas na área de biossensores. No entanto, esses biossensores baseados em nanomateriais também enfrentam alguns desafios. Por exemplo, nanomateriais derivados de metais e semicondutores e pontos quânticos geralmente contêm metais pesados tóxicos (ou seja, cádmio, chumbo)(LEE; SURESH; EKPENYONG, 2019), limitando significativamente suas aplicações de biossensores devido a riscos potenciais e altos custos. Os nanomateriais dopados com corante sintéticos ou outras substâncias não naturais não são apenas prejudiciais ao meio ambiente, mas também sujeitos a fácil degradação, afetando sua precisão de detecção (YANG *et al*., 2019). À luz desses desafios na aplicação de nanomateriais tradicionais para biossensor, esforços significativos têm sido dedicados ao desenvolvimento de nanomateriais com melhor biocompatibilidade e fotoestabilidade e menor toxicidade, mantendo as excelentes propriedades ópticas e eletroquímicas. Nesse contexto, os *Carbon dots* têm recebido enorme atenção em biossensores (SONG *et al*., 2016; ZHANG *et al*., 2018).

2.4.1 Pontos Quânticos (PQ)

Os PQs são um novo membro da família de nanomateriais à base de carbono de dimensão zero com diâmetros inferiores a 10 nm, conhecidos por suas propriedades exclusivas de fluorescência. PQs foram descobertos por acaso por Xu *et al*. ao purificar nanotubos de carbono de parede simples (SWCNTs) por eletroforese (XU *et al*., 2004). Desde então, os PQs foram desenvolvidos rapidamente e se tornaram um dos principais pontos de pesquisa em ciência dos materiais (JI *et al*., 2020; PENG *et al*., 2017; ZHENG *et al*., 2020). Com base na natureza do precursor de carbono, estrutura central e efeito quântico, os PQs podem ser divididos em três categorias: ou seja, pontos quânticos de grafeno (GQDs), nano pontos de carbono (CNDs) e pontos quânticos de carbono (CQDs). GQDs são nanoestruturas em forma de disco conjugadas com π, que são geradas principalmente pelo corte de grandes estruturas de grafeno (CAYUELA *et al*., 2016).

PQs têm uma área de superfície rica e contêm um grande número de grupos funcionais que podem ser usados para modificar os receptores biológicos. Ao mesmo tempo, os PQs também são bons condutores eletrônicos que podem realizar a rápida condução de elétrons entre a interface de detecção e os eletrodos. Devido à sua excelente capacidade de

transporte de elétrons, são frequentemente usados em biossensores eletroquímicos, pois podem interagir diretamente com os analitos alvo para influenciar os sinais eletroquímicos dos eletrodos (JI *et al*., 2020). Contudo, os materiais de partida para a síntese de PQs podem apresentar alto custo e baixo rendimento de produção. Portanto, a busca por métodos que envolvam solventes não tóxicos e materiais de partida mais ecológicos e econômicos são uma preocupação fundamental (GOSWAMI *et al*., 2022).

2.4.2 Síntese de Carbon dots a partir de resíduos vegetais

A síntese de CDs utilizando como fonte resíduos vegetais, animais ou industriais tornou-se foco de interesse dos pesquisadores. Esses materiais residuais são baratos e disponíveis em geral, e o reaproveitamento de recursos naturais tem o potencial de reduzir os poluentes e seus impactos ambientais (DE OLIVEIRA; DA SILVA ABREU, 2021). Diversas partes vegetais podem ser utilizadas como fonte de CDs, como cascas (SURENDRAN; RAMALINGAM, 2019) frutas (PRASANNAN; IMAE, 2013), flores (HIMAJA; KARTHIK; SREEDHAR, 2014), raízes (LIU *et al*., 2019) e amêndoas (GANAPATHI; BALASUBRAMANIAN; CHINNASAMY, 2023).

Muitas fontes alternativas de carbono podem ser usadas para sintetizar CDs. Geralmente, a fonte de carbono verde utilizada provém de resíduos não utilizados, mas de alto valor (DAULAY et al., 2024). A utilização de resíduos vegetais que antes iriam ser descartados no ambiente causando grande acúmulo de lixo e contaminação, tornou-se uma estratégia promissora para síntese de nanomateriais. Resíduos orgânicos possuem um grande potencial para estudos devido as características físico-químicas do material de origem (DE OLIVEIRA; DA SILVA ABREU, 2021).

Vários materiais naturais e artificiais têm sido utilizados como precursores para a síntese de CDs. É necessário usar precursores de carbono verde para sintetizar CDs ecológicos que não sejam tóxicos, sejam baratos e sejam aproveitados como resíduos.

2.4.3 Síntese de Carbon dots a partir da amêndoa do Pequi

O pequi (*Caryocar coriaceum*) é uma espécie arbórea nativa do cerrado brasileiro muito utilizada para alimentação devido suas características nutricionais. O consumo desse fruto se faz principalmente da polpa, tanto *in natura* quanto para extração do óleo, sendo muitas vezes a amêndoa descartada, já que ela se encontra protegida pelo caroço e o processo para retirada ocorrer de forma muito precária, sendo dificultada pelos espinhos, gerando um

alta produção de resíduos (OLIVEIRA; SCARIOT, 2010). Os caroços despolpados, que são descartados na produção da polpa em conserva, polpa congelada, obtenção do óleo da polpa, e aqueles provenientes do descarte do fruto inteiro, podem ser aproveitados para a retirada da amêndoa interna (CARRAZZA, D'ÁVILA, 2010).

A amêndoa do pequi se destaca por suas características físico-químicas, apresentando um alto teor de lipídeos, como também carboidratos e proteínas (OLIVEIRA *et al*., 2010), o que pode representar uma fonte promissora para síntese de novos materiais (DE OLIVEIRA; DA SILVA ABREU, 2021; SURENDRAN; RAMALINGAM, 2019).

No Brasil anualmente se desperdiça milhões de toneladas de alimentos e resíduos aproveitáveis nas indústrias de alimentos. Durante o processamento de frutos de pequi, diferentes resíduos, como cascas, sementes, caroços ou bagaço, não são aproveitados (CAMPOS, et al, 2016). Devido à complexidade apresentada pela estrutura morfológica do caroço do pequi, que apresenta uma grande quantidade de espinhos, o resíduo é descartado no meio ambiente (assim, a castanha acaba sendo descartada com o caroço) de forma incorreta acarretando problemas de contaminação ambiental, principalmente nos recursos hídricos e no solo (GUIMARÃES, 2015). Os caroços despolpados, que são descartados na produção da polpa em conserva, polpa congelada, obtenção do óleo da polpa, e aqueles provenientes do descarte do fruto inteiro, podem ser aproveitados para a retirada da amêndoa interna (CARRAZZA, D'ÁVILA, 2010), desta forma o aproveitamento da amêndoa representaria mais uma fonte de renda para economia local.

3 OBJETIVOS

3.1 Geral

Desenvolver e avaliar o desempenho de um imunossensor eletroquímico para detecção de *Salmonella* sp. em alimentos e águas residuais derivadas de alimentos, utilizando pontos quânticos de pequi como marcador eletroquímico.

3.2 Específicos

1) Sintetizar pontos quânticos a partir da amêndoa do pequi.

2) Caracterizar os pontos quânticos de pequi.

3) Realizar estudos de bioconjugação de pontos quânticos ao anticorpo IgG (Imunoglobulina G).

4) Otimizar as etapas de montagem e funcionamento do biossensor para detecção de *Salmonella* sp.

5) Caracterizar o biossensor por meio de técnicas eletroquímicas e morfológica.

6) Caracterizar a resposta analítica do biossensor.

7) Avaliar o desempenho do biossensor em amostras de alimentos e águas residuais derivadas de alimentos, intencionalmente contaminadas com *Salmonella* em laboratório.

4 ARTIGOS

4.1 Biosensors: monitoring and identification of microorganisms in wastewater

BIOSENSORS: MONITORING AND IDENTIFICATION OF MICROORGANISMS IN WASTEWATER

Rachel Menezes Castelo[a], Castelo, R. M. rachel.menezes@aluno.uece.br

Luana Carvalho da Silva[a], Silva, L. C. lu_luanacarvalho@hotmail.com

Joana de Barros Alexandre[a], Alexandre, J. B. joana.alexandre@aluno.uece.br

Gabrielle Albuquerque Freire[a], Freire, G. A. gabi.a.freire@hotmail.com

Terezinha Feitosa Machado[b], Machado, T. F. terezinha.machado@embrapa.br

Roselayne Ferro Furtado[b*], Furtado, R. F. roselayne.furtado@embrapa.br

Carlucio Roberto Alves[a], Alves, C. R. carlucio.alves@uece.br

[a]*Science and Technology Center, University of Ceará (UECE), Fortaleza, CE, Brazil.*

[b]*Embrapa Tropical Agroindustry (CNPAT), Brazilian Agricultural Research Corporation (EMBRAPA), Fortaleza, CE, Brazil*

***Corresponding author:** roselayne.furtado@embrapa.br (FURTADO, R. F.)

Abstract: Waterborne microbial diseases cause mortality and morbidity worldwide. The presence of disease-causing pathogens in wastewater can provide an excellent diagnostic tool for infectious diseases. Wastewater from domestic sources and the food industry offers favorable conditions for the growth of different microorganisms, and treatment plants are often inefficient in decontamination treatment. The search for methods for monitoring and identifying pathogenic microorganisms are current research topics, and these can be used to monitor possible contamination through water consumption. In addition to sensitivity, these methods must be fast and specific for the potability of the water. This article provides an overview of the primary analysis methods used in monitoring pathogenic microorganisms in wastewater, highlighting the use of biosensors, as well as the advantages and main challenges of these methods.

Keywords: pathogenic microorganisms; wastewater; detection; methods.

1 INTRODUCTION

Water pollution is one of the causes of water scarcity for human consumption and decline in aquatic biodiversity. Although the availability of fresh water has decreased in recent decades, water demand continues to increase, especially in areas with low precipitation rates (MEKONNEN; HOEKSTRA, 2016; SANTOS et al., 2023). These are recipients of domestic, industrial, and agricultural sewage and, consequently, a vehicle for the environmental dispersion of pollutants. Approximately 80% of the world's wastewater is discharged, without treatment, into lakes, polluting rivers, the environment, and oceans (DUTT et al., 2020). Wastewater is understood as water resulting from domestic, urban, industrial, or service activities; surface runoff; rainwater from unitary or pseudo-separate drainage systems, or any accidental inflow or infiltration into wastewater drainage systems (Decree-Law No. 119/2019, of August 21).

Wastewater characteristics comprise its physical appearance, chemical parameters, and abundance of microorganisms. The composition and concentration of substances in wastewater vary widely and can be classified into three broad categories: microorganisms, inorganic chemicals, and organic pollutants (EJEIAN et al., 2018; ZHANG et al., 2020). Currently, wastewater is considered a significant source of pollution, which can cause a series of risks to environmental and human health (ZHANG et al., 2020; SANTOS et al., 2023). The health risks induced by these pollutants raise widespread concerns. The precondition for accurately assessing these health risks is to analyze the types and concentrations of these pollutants in wastewater (MAO et al., 2020; YAROSHENKO et al., 2020)

Pathogenic microorganisms are frequently found in wastewater, even after water treatment (MEDEMA et al., 2020a; VOIGT et al., 2020). Efficient monitoring prevents and controls infectious diseases (MAO et al., 2020). By analyzing biomarkers of contagious diseases in wastewater collected from specific collection points, it is possible to monitor disease transmission in certain areas in near-real time comprehensively (MAO; ZHANG; YANG, 2020; OLIVENZA; CASADESÚS; ANSALDI, 2020; RANDAZZO et al., 2020; YANG et al., 2020; KADADOU et al., 2022).

In recent decades, there has been considerable demand for research to produce robust and efficient technologies with low operational and energy costs for detecting chemical and biological contaminants. Recent technological advances in biosensors and spectroscopy have quantitatively and qualitatively improved detection sensitivity (LAAD AND GHULE, 2023).

The effectiveness of a biosensor is assessed by the specificity of the probe-analyte and the sensitivity of the biophysical interactions of the analyte-probe intercepted by the transduction system. The detection mechanism is based on electrochemical, calorimetric, optical, and acoustic configurations characterized by differences in the measurable perspectives of probe-analyte combinations (MALIK et al., 2024).

Large amounts of freshwater could be reused from industrial water recycling, reducing costs and environmental pollution (BURGESS et al., 2015; KADADOU et al., 2022). Reuse contributes to the supply of industrial water, which may be threatened in the future due to the increased demand for freshwater from other sectors, including agricultural use and drinking purposes. Because of this, this article provides an overview of the main analytical methods used in monitoring pathogenic microorganisms in wastewater, highlighting the performance factors of biosensors, as well as the advantages and main challenges regarding these methods.

2 WASTEWATERS: TREATMENT PLANT AND EPIDEMIOLOGICAL STUDY

Water quality assessment is an integral part of wastewater treatment plant operation, as it provides essential data to assess the level of treatment required to ensure human health and ecological safety (DO et al., 2020). Given their nature, microbiological parameters require samples to be collected punctually for monitoring and monitoring.

Wastewater Treatment Plants are infrastructures designed to reduce the pollutant load of wastewater so that the discharge of treated effluent does not affect emissions in the receiving environment and are, therefore, essential instruments for environmental protection (MARECOS DO MONTE et al., 2016). These plants treat wastewater of domestic and industrial origin so that it can be safely discharged into the sea or river with pollution levels that are acceptable for the environment (MOREIRA, 2014).

Conventional treatment is also known as a complete cycle because it covers seasonal variations that affect water and consists of five stages: coagulation, flocculation, decantation, filtration, and disinfection. In wastewater treatment plants, water is subject to a series of processes and operations that aim to restore the quality it had when collected for supply or when supplied for supply, with the documentation of the last stage being responsible for reducing the number of pathogenic microorganisms using mainly chlorine, UV or ozone (SILVA; DANIEL, 2015). This reduction is necessary when the objective is to protect public

health associated with its discharge into the receiving environment or when the aim is reuse (MARECOS DO MONTE et al., 2016).

Studies based on Wastewater Epidemiology (WWE) present a new epidemiological tool that has the potential to act as a complementary activity to current infectious disease surveillance systems and an early warning system for disease outbreaks (SIMS; KASPRZYK-HORDERN, 2020). Wastewater is characterized by containing a high amount of pathogenic organisms, such as total coliforms, fecal coliforms, *E. coli*, and the eggs of intestinal parasites, which can be harmful to the receiving body and generate several impacts on the environment, such as the risk of spreading infectious diseases (KADADOU et al., 2022).

By detecting pathogenic microorganisms in wastewater effluents, sources of human contamination can be suggested, and information on waterborne diseases can be obtained. Resistant bacteria (GOULIOURIS et al., 2019; VOIGT et al., 2020) and viruses stand out as risk factors for infectious diseases due to their high mutation rates and ability to adapt to a new host, such as humans (MEDEMA et al., 2020b; TEIXEIRA et al., 2020).

3. PATHOGENIC MICROORGANISMS

Pathogens are organisms capable of causing disease in a host. In addition to bacteria, pathogens include viruses, fungi, protist and helminths (Table 1) (ABU-ORF et al., 2014). One of the primary sources of water contamination is the discharge of domestic, industrial, or agricultural wastewater, which has not been treated appropriately, constituting a potential focus for spreading diseases due to pathogenic organisms (DE SOUZA et al., 2015).

Table 1. Main microorganisms causing infections present in wastewater.

	Microrganisms	Related infection
Bacteria	*Escherichia coli*	Gastroenteritis
	Salmonela spp. (non-typhoid)	Salmonellosis
	Salmonela typhi	Typhoid
	Shigella spp.	Shigellosis

	Vibrio cholerae	Cholera
Virus	Astrovírus	
	Calicivírus	Gastroenteritis
	Coronavírus	
	Vírus entérico	Paralysis, gastroenteritis, fever and herpangina
	Vírus da hepatite A	Infectious hepatitis
	Parvovírus	
	Rotavírus	Gastroenteritis
Protists	*Cryptosporidium parvum*	Cryptosporidiosis
	Entamoeba histolytica	Amebiasis
	Giardia lamblia	Giardiasis
Helminths	*Ascaris lumbricoides*	Ascariasis
	Schistosoma mansoni	Schistosomiasis
	Trichuris trichiura	Trichuriasis

Source: (QASIM; ZHU, 2018)

Due to their high infection rates, facultative pathogenic bacteria of the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter ssp*.) and *Escherichia coli* are critical for human public health. In addition, these bacteria frequently transmit or acquire plasmids that can lead to high levels of antibiotic resistance (MAGIORAKOS et al., 2011; VOIGT et al., 2020).

Current regulations and legislation require critical review to determine the safety of alternative water sources and their reuse as part of the solution to the global water crisis (TEIXEIRA et al., 2020). The best methods for monitoring pathogens should be rapid, highly sensitive, selective, and distinguish between viable and non-viable organisms. Combining methods can be advantageous for lower detection limits, analysis of samples in a shorter time and possibilities for more efficient treatment of wastewater (MOSHER et al., 2016).

Effective detection methods for analysis of water parameters are identified by chemical or laboratory checks performed by stationary devices, are slow and time-consuming procedures (SHABAN; EDDAIF; TELEGDI, 2023). The types of sensors developed, namely chemical, electrochemical, and biosensors, have gained particular attention for early detecting contaminants. Sensors are classified according to their applications and transducers. Although PCR, cell culture, colony counting, and immunology-based methods are reliable and accurate, they are often expensive and more time-consuming (KADADOU et al., 2022).

4. METHODS FOR IDENTIFYING AND MONITORING MICROORGANISMS

The detection of pathogenic microorganisms can be performed using several methods, including techniques based on immunology (HUANG et al., 1992; SUE et al., 2014), molecular biology (ADAMU et al., 2020; TEIXEIRA et al., 2020), mass spectrometry (VOIGT et al., 2020; YANG et al., 2020) and biosensors (MENDOZA et al., 2020; CALISKAN-AYDOGAN et al., 2024). Although these methodologies vary in operational principles and response times, they all demonstrate high selectivity in identifying specific microorganisms. Innovative methods have emerged with technological advancement, offering greater sensitivity, selectivity, and reduced response times. These improvements are essential for the early detection, the continuous monitoring, and the effective prevention of infections, enabling rapid and accurate interventions at early stages.

Biosensors have gained prominence due to their high specificity, selectivity, low limit of detection (LOD), fast response time, and the possibility of on-site testing (CALISKAN-AYDOGAN et al., 2024; SEO et al., 2023; MUHSIN et al., 2022). Another essential advantage of biosensors is the possibility of using a specific antibody as a pathogen recognition element, enabling the identification of microorganisms, as well as the possibility its quantification (MUHSIN et al., 2022; GUERRERO-ESTEBAN et al., 2022).

4. 1 BIOSENSORS

A biosensor is defined by IUPAC (International Union of Pure and Applied Chemistry) as an analytical device that integrates a biologically derived recognition of molecules such as enzymes, antibodies, phages, aptamers, or single-stranded DNA with a suitable physicochemical transducer, such as electrochemical, optical, thermometric, piezoelectric and magnetic (IQBAL et al., 2000).

Biosensors provide real-time identification of microbiological contamination, while traditional techniques take several days to obtain the result (EJEIAN et al., 2018; YAROSHENKO et al., 2020). Many portable devices are designed to amperometrically monitor various microorganisms by deoxidation or reduction reactions (KOKKINOS; ECONOMOU, 2017). According to Qasim and Zhu (2018), the most important pathogenic bacteria related to water consumption are *Salmonella sp., Shigella sp., Vibrio cholerae, and Escherichia coli.*

The application of biosensor-based strategies to detect microorganisms in water has become a promising research field, as described in Table 2, taking into account the response time and detection limit.

Table 2. Pathogenic microorganisms identified in water using biosensors.

Method	Sample types	Microorganisms (Identification of genus and/or species)	Response time	Limit of detection	References

Biosensor with oligonucleotides	Tap water	*Escherichia coli*	1 h	10^3 CFU/mL	Caliskan-Aydogan et al. (2024)
Biosensor with recognition proteins	Lake waters	*Salmonella*	20-45 min	9 CFU/mL	Liu et al. (2024)
Biosensor with DNA primers	Tap water	Cianobactérias	20 min	9,99 pg/mL	Seo et al. (2023)
Immunosensor	Tap water	*Salmonella* *E. coli O157:H7* *Legionella*	30-40 min	3 CFU/mL	Muhsin et al. (2022)
Immunosensor	Wastewater, river and urban waters	SARS-COV-2	60 min	1,2 pg/mL	Guerrero-Esteban et al. (2022)
Biosensor	Wastewater	Bacteriófagos	-	1,6 PFU/100 ml.	(Olivenza; CASADESÚS; ANSALDI, 2020)
Immunosensor	Drinking water	*Escherichia coli*	1 h	3×10^1 CFU/mL	(CIMAFONTE et al., 2020)
Biosensor	Wastewater (popular beach)	*Escherichia coli*	20 min	4×10^3 CFU/100 mL.	(JÕGI; VÄLING; RINKEN, 2020)

Electrochemical sensor	Wastewater	*Escherichia coli*	≤ 5 min	8,7 ± 0,5 CFU/mL	(MUGO; LU; DHANJAI, 2020)
Biosensor	Wastewater and laboratories	*Escherichia coli*	2 h	10^4 CFU/mL	(ZAOURI et al., 2019)
Biosensor	Wastewater (seawater from coastal areas)	*Escherichia coli*	75 min	5 CFU/mL	(BRICIU-BURGHINA et al., 2019)
Biosensor	Wastewater	*Escherichia coli*	1 h	6 CFU/mL	(ZUSER et al., 2019)
Biosensor	Wastewater	Células bacterianas	45 min	1,9 x 10^3 CFU/mL.	(RENGARAJ et al., 2018)

Caliskan-aydogan and Alocilja (2024) developed an oligonucleotide biosensor for detecting KPC-producing *E. coli* from tap water and complex food samples using carbohydrate-coated magnetic and gold nanoparticles. The detection was successful in detecting 10^3 CFU/mL in 1 h. Liu (2024) used a long-tailed fiber protein from phage SEP37 in *E. coli* as a biorecognition element to construct a biosensor. The biosensor rapidly detected *Salmonella* in lake water samples, with a limit of detection 9 CFU/mL.

Seo et al. (2023) proposed a rapid electrochemical biosensor composed of DNA primer/iridium nanoparticles to detect *Aphanizomenon flos-aquae* in freshwater. An alternating current electrothermal flow technique was implemented to rapidly detect the target, which could reduce the detection time to 20 min. The target gene was detected at a concentration of 9.99 pg/mL in tap water, and the detection range was from 0.1 ng/mL to 10^3 ng/mL with high selectivity. Muhsin et al. (2022) investigated a microfluidic biosensor for a

rapid and simultaneous detection of *Salmonella, Legionella*, and *Escherichia coli* O157:H7 in tap water and wastewater. The results demonstrated that the biosensors could detect *Salmonella, Legionella*, and *E. coli* 0157:H7 simultaneously with a limit of detection 3 bacterial cells/mL in 30-40 min.

Guerrero-Esteban et al. (2022) reported a new method for detecting SARS-CoV-2 in wastewater based on an electrochemiluminescence immunosensor amplified by carbon nanodots for the determination of the SARS-CoV-2 Spike S1 protein. The immunosensor demonstrated high specificity in the presence of other proteins related to the virus and responded linearly to the concentration of SARS-CoV-2 Spike S1 over a wide range, with a limit of detection 1.2 pg/mL. Detection of SARS-CoV-2 Spike S1 in urban wastewater can be used to measure virus circulation in the population and detect a possible resurgence of COVID-19.

Mugo; Lú; Dhanjai (2020) examined a versatile pathogen-imprinted polymer electrochemical sensor for *Escherichia coli* detection. The sensor is based on the layer-by-layer assembly of multi-walled carbon nanotubes and nanocellulose films integrated with polyaniline-doped phenylboronic acid. The sensing layer is a pathogen-imprinted microcontact polymer based on poly(methacrylic acid). The sensor exhibited high specificity for *E. coli* in real biological matrices while effectively discriminating *E. coli* among other bacteria. Using capacitance and impedance as transduction methods, the sensor recorded a low limit of detection 8.7 ± 0.5 CFU/mL, with a fast response of ≤ 5 min.

Cimafonte et al. (2020) reported the achievement of a simple and low-cost electrochemical impedance immunosensor based on a screen-printed gold electrode for rapidly detecting *Escherichia coli* in water. The immunosensor is fabricated by covalently immobilizing anti-*E. coli* antibodies on a gold surface by the photochemical immobilization technique, a simple procedure capable of vertically binding antibodies to gold surfaces. An amplification step was included in the experimental method to improve the signal. The response enhancement was achieved by transporting anti-*E. coli* (25 μg mL^{-1}) into the microfluidic cell for 30 min. Introducing this standard amplification procedure led to a significant increase in the impedance enhancement, which allows measuring *E. coli* in drinking water with a limit of detection 3×10^1 CFU/mL in only 1 h.

Olivenza et al. (2020) designed a highly sensitive epigenetic bacteriophage biosensor, allowing the detection of up to 1.6 phages/100 ml and the identification of the bacterial

receptor. The study described two versions of biosensors capable of detecting phages using lipopolysaccharide (LPS) or FhuA protein as a receptor. Additional variants of this biosensor can be designed depending on the type of phage to be detected and quantified.

Rengaraj et al. (2018) developed an impedimetric biosensor to identify bacteria in wastewater. Considering its remarkable simplicity, cost-effectiveness, and biodegradability, the sensor demonstrated a response time of 45 min with a limit of detection 1.9 x 10^3 CFU/mL. It is proving to be an attractive solution for portable testing that addresses the challenges of traditional, time-consuming, and expensive laboratory-based analyses. Rapid and on-site screening of pathogenic microorganisms in drinking water can help to reduce this number markedly.

Recent studies on biosensors for the detection of *Escherichia coli* in wastewater, seeking different standardizations in the assemblies to improve the analyte's response time and detection limit, are reported in the literature. Zuser et al. (2019) combined cyclic voltammetry with immunomagnetic separation (magnetic core/ shell nanoparticle/ immunofunctionalized polyaniline) to obtain a response in approximately 1 h with a limit of detection 6 CFU/mL. Briciu-Burgmina et al. (2019) used β-glucuronidase, an *E. coli* biomarker enzyme, as a fluorogenic substrate to improve detection, providing results in only 75 min with a limit of detection 5 CFU/mL.

The physicochemical properties of the interface, the biomolecules, and also the surface on which they will be immobilized in the device are of paramount importance and must be considered in the planning stage of a biosensor (CHAUDHARY, et al., 2021). Considering the context of the use of antibodies, one of the biggest challenges in their immobilization is maintaining their immunological activity, particularly their specificity. Because of this, some immobilization methods share such factors to increase the performance of the immunosensor according to its application (VASHIST; LUONG; 2018).

4.2 OTHER METHODS

4.2.1 CULTURE-BASED METHOD

In identifying microorganisms, culture-based methods are considered the oldest and most traditional since they are based on microorganisms morphological, metabolic, and physiological differentiation (WETLER-TONINI, REZENDO, AND GRATIVOL, 2011). The culture method has been the gold standard since the discovery of enteric fever and the etiological agent in 1880 (ANDREWS et al., 2015). This traditional isolation method involves

enumerating the target bacteria according to their unique morphological and biochemical characteristics, standardized by several regulatory agencies (LEE et al., 2015). In general, the guidelines proposed by regulatory agencies are essentially similar and involve four main steps: non-selective pre-enrichment, selective enrichment, plating on selective isolation agar, and biochemical and serological tests (LEE et al., 2015; GAST et al., 2020).

Culture methods have been widely used, especially in laboratory routines. Although inexpensive and useful for obtaining quantitative and qualitative information about various microorganisms present in a sample, culture methods are laborious, time-consuming, and consume a significant number of materials due to the steps of analysis. In addition, it is essential to highlight that the results are only collected after a few days, with the possibility of false positives occurring and that these methods cannot identify non-culturable cells (FRANCO-DUARTE, 2019).

Furthermore, to identify microorganisms isolated using the culture method, it is necessary to use other techniques, such as molecular identification. For example, Ake et al. (2023) used the culture method to isolate microorganisms from tannery wastewater. The results indicated that the effluents contained bacteria, yeasts, and fungi. After growth and isolation, the microorganisms underwent the molecular identification stage, which showed that the isolated strains were the bacteria *Bacillus licheniformis* and *Bacillus megaterium*, the fungi *Byssochlamys* sp., and *Candida maltosa*. El-Liethy, Hemdan, and El-Taweel (2023) monitored industrial wastewater bacteria capable of degrading textile wastewater dye. The authors used the culture method followed by phenotypic and genotypic identification and identified *Alkalibacterium pelagium, Enterobacter kobei*, and *Chryseobacterium montanum*.

4.2.2 MASS SPECTROMETRY

Mass spectrometry is an analytical method in which chemical compounds are ionized in related molecules, and the ratio of their mass to be beneficial (m/z) is measured. Although mass spectrometry was discovered in the early 2000s, its scope was limited to the chemical sciences (SINGHAL et al., 2015). Since the advent of matrix-assisted laser desorption/ionization (TOF-MS), time-of-flight mass spectrometry (MALDI) is used as a tool for microbial characterization. The speed and effectiveness of the approach have suggested applications against bioterrorism, prevention of food contamination, and monitoring the spread of antibiotic-resistant bacteria (SANDRIN; GOLDSTEIN; SCHUMAKER, 2011).

MALDI-TOF MS has gained popularity as a microbial biotyping tool due to its speed, low cost, simplicity, and applicability to various microbes. It is becoming an increasingly essential technique for microbial characterization and identification in environmental microbiology and microbial diversity studies (KOPCAKOVA et al., 2014; PATIL et al., 2015). During the process, microbes are identified using intact cells or cell extracts. The process is rapid, sensitive, and economical regarding human resources and the costs involved.

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Two techniques are used to analyze microorganisms by MALDI-TOF MS: digital rain recognition and biomarker assignment. Although digital rain recognition, which involves comparing the obtained mass spectra with a database of spectral patterns, it is frequently used. Species and strains can be difficult to discriminate if they have similar digital rain spectral patterns. On the other hand, several biomarker assignment approaches focusing on specific spectra have been reported (HOLLAND et al., 1996; ULRICH et al., 2019).

MALDI-TOF MS is a fast, accurate, and reliable approach for identifying bacteria compared to conventional phenotypic techniques or molecular methods (SINGHAL et al., 2015). Table 3 highlights some studies using the technique and the microorganisms identified.

Table 3. Pathogenic microorganisms identified in wastewater by the MALDI-TOF MS technique.

Technique	Sample types	Microorganisms (Identification of genus and/or species)	References
MALDI-TOF (MS) e *carbon dots* (CDs)	Samples of treated water and sewage	*Escherichia coli* O157: H7	(YANG et al., 2020)

MALDI-TOF	Drinking water reservoir subject to discharge from two sewage treatment plants	Antibiotic resistant bacteria (belonging to the ESKAPE group and *Escherichia coli*)	(VOIGT et al., 2020)
MALDI-TOF	Treated and untreated wastewater from 20 treatment plants	*Enterococcus faecium* (antibiotic resistant)	(GOULIOURIS et al., 2019)
MALDI-TOF	Effluents from urban wastewater treatment plants	*Escherichia coli* and *Enterococcus.*	(PICCIRILLI et al., 2019)
MALDI-TOF	WWTP in petrochemical industry	Denitrifying bacteria	(ANTUNES; BALLARINI; VAN DER SAND, 2019)
MALDI-TOF	Treated effluent and surface water close to upstream and downstream of 50 WWTPs	Carbapenemase-producing bacteria (CEC) – multidrug-resistant organisms	(MATHYS et al., 2019)
MALDI-TOF	Hospital wastewater from sewage	*Escherichia coli* *Klebsiella oxytoca, Citrobacter freundii, Enterobacter sp. Pseudomonas aeruginosa*	(HUTINEL et al., 2019)
MALDI-TOF	Wastewater, surf water and wastewater treatment sources	*Escherichia coli;* *E. coli* with resistance to ESBL; *Klebsiella pneumoniae* carbapenemase (KPC).	(HABERECHT et al., 2019)
MALDI-TOF e PCR (Polimerase Chain Reaction)	Cow dung water (sewage sludge)	*Enterococcus faecium*	(LAUKOVÁ et al., 2019)

MALDI-TOF e PCR (Polimerase Chain Reaction)	Wastewater treatment plant	Bacteria *(Enterobacter asburiae; Sphingobacterium griseoflavum; Chryseobacterium bovis)* Yeasts (*Hanseniaspora uvarum; Wicker-hamomyces anomalus; Torulaspora delbrueckii*). Filamentous fungi (*Fusarium oxysporum*).	(PIRES et al., 2017)
MALDI-TOF e PCR (Polimerase Chain Reaction)	Two wastewater treatment plants	*E. coli* e *K. pneumoniae* [positive for mcr-1 (antibiotic resistance gene)]	(OVEJERO et al., 2017)

WWTP means Wastewater Treatment Plant

Gouliouris et al. (2020) analyzed treated and untreated wastewater from 20 municipal treatment plants in East England. Bacteria were identified at the species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI - TOF MS). Vancomycin-resistant *Enterococcus faecium* was isolated from 20 treatment plants. Only three of the 20 WWTPs could eliminate the microorganism from the medium due to the ultraviolet light disinfection process. The remaining (17 WWTPs) released the bacteria into the medium after the decontamination process was used, demonstrating evidence of contamination in the treated water from most of the treatment plants monitored in the study.

In studies by Haberecht et al. (2019), they described colonies of *E. coli* with the production of Extended Spectrum Beta-Lactamase (ESBL) and *Klebsiella pneumoniae* carbapenemase (KPC) in wastewater treatment sources in Northern Colorado, USA. Using MALDI - TOF MS, they identified that the total abundance of *E. coli* decreased with the water treatment process as expected, but the percentages of *E. coli* with ESBL resistance increased (1.70%) in surface water. Studies like this can help prevent the spread of drug-resistant *E. coli* in wastewater, avoiding the risk of contamination in various animals and humans.

Yang et al. (2020) detected pathogenic bacteria using a pH-sensitive sensor and fluorescent carbon dots to identify *Escherichia coli* O157:H7 by MALDI-TOF MS. They obtained an LOD of 1 CFU/mL. The results were entered into a database and bacterium was identified by comparing the ion mass and relative strength of each specific peak with the reference spectra in the specification database. Voigt et al. (2020) screened bacteria from the

SKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp*) and *E. coli* in a water reservoir subject to discharge from two sewage treatment units. The presence of these bacteria in the medium was identified, even after water treatment.

The technology's limitation is that the identification of new isolates is only possible if the spectral database contains hundreds of mass fingerprints of peptides from strains of specific genera/species/subspecies/strain types (SINGHAL et al., 2015). This places a high demand for analyses to feed the database microorganisms.

4.2.3 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) technique uses the nucleotide sequence as a "primer" to amplify the target gene (such as a specific DNA sequence of pathogenic microorganisms) by a series of chain reactions. Several new techniques have been derived based on PCR, such as reverse transcription-PCR (RT-PCR). Since many pathogenic viruses in wastewater are RNA viruses, the viral RNA in the sample must be transcribed into cDNA (DNA synthesized from an mRNA template), and then the cDNA can be used as the target sequence for the PCR amplification reaction. The abundance of viruses is detected based on the specific amplified DNA product (ZHANG et al., 2020).

PCR techniques are fast, sensitive, and highly specific. However, a PCR occurrence can be easily impaired by other substances existing in the system. For example, humic acid, fulvic acid, specific ions, and carbohydrates can interfere with the action of Taq polymerase (FARHADKHANI et al., 2020; YANAGIMOTO et al., 2020; ZHANG et al., 2020).

Some compounds used for concentration, storage, and purification of wastewater samples cause inhibitors, such as EDTA, sodium lauryl sulfate, and some mercapto compounds. It is worth noting that the method cannot distinguish between live and dead cells. Furthermore, it is impossible to determine whether virions are infectious in water samples using PCR techniques (RAJAPAKSHA et al., 2019; ZHANG et al., 2020). However, a wide variety of microorganisms can be identified (Table 4), making this method one of the most specific in terms of analysis.

Table 4. Pathogenic microorganisms identified in wastewater using the Polymerase chain reaction (PCR) technique.

Method	Sample types	Microorganisms (Identification of genus and/or species)	References
PCR	Untreated wastewater, tertiary treated wastewater and surface water.	Norovirus (NoV) e Hepatitis A (HepA).	(TEIXEIRA et al., 2020)
PCR	Wastewater treatment plants (WWTP)	SARS-CoV-2	(MEDEMA et al., 2020b)
RT-PCR in real time.	Six wastewater treatment plants (WWTPs)	SARS-CoV-2	(RANDAZZO et al., 2020)
PCR digital droplet (ddPCR). Compared to traditional quantitative PCR (qPCR)	Untreated wastewater.	Norovirus (GI/GII) e adenovirus.	(JAHNE et al., 2020)
PCR	Wastewater treatment plant	*Escherichia coli* (antibiotic resistant).	(ADEGOKE et al., 2020)
PCR	Excavated well and tap.	*Salmonella* species (antibiotic resistant)	(ADAMU et al., 2020)
RT-PCR in real time.	Treated wastewater used in irrigation.	*Campylobacter risk*	(FARHADKHANI et al., 2020)
PCR and agar disc diffusion.	Treated wastewater used in irrigation.	*Escherichia coli* enterotoxigenic - ETEC (multiresistant).	(CHIGOR et al., 2020)
PCR	Wastewater	*Escherichia coli* enteropathogenic (EPEC)	(EDITH CHÁVEZ-BRAVO et al., 2020)
PCR	Wastewater	*Escherichia coli* (extended spectrum beta-lactamase producer)	(ADEKANMBI et al., 2020)

qPCR	Wastewater, recycled water, tap water and fresh water.	Total and fecal coliforms (antibiotic resistant)	(ROCHA et al., 2019)
RT-PCR in real time.	Wastewater treatment plant.	Enteroviruses and Noroviruses	(SIMHON et al., 2019)
RT-PCR in real time	Wastewater treatment plant.	*Salmonella* Species	(YANAGIMOTO et al., 2020)

Teixeira et al. (2020) performed a quantitative polymerase chain reaction (PCR) (qPCR) detection of enteric virus genogroups I (GI) and II (GII) and Hepatitis A (HepA), and FIB (E.coli, enterococci, and fecal coliform). The developed method was suitable for various water devices - groundwater, surface, treated, and untreated drinking water, allowing its use in routine water quality to detect and quantify NoV GI/GII and HepA viruses, enabling to more accurately assess of potential public health risks.

In the studies by Jahne et al. (2020), droplet digital polymerase chain reaction (ddPCR) quantification analyses of viral enteric pathogens (norovirus genogroups GI and GII and human adenoviruses) were performed in untreated wastewater and combined wastewater. The results emphasize the unique quality of on-site wastewater and enable quantitative assessment of microbial risks from decentralized water reuse.

Chigor et al. (2020) investigated treated wastewater used to irrigate fresh produce in Nsuskka, southeastern Nigeria, as a reservoir of enterotoxigenic and multidrug-resistant *Escherichia coli*. The study concluded that treated sewage, irrigated soils, and vegetables harbored enterotoxigenic *E. coli* (ETEC) strains were detected by conventional PCR assays and thus were unsuitable for domestic use and irrigation. The study demonstrated the need for regular monitoring of sewage treatment plants to prevent ETEC-related diseases and to determinate effective intervention strategies.

One of the most apparent limitations of PCR techniques comes from the lack of differentiation between viable and non-viable cells. This is because genetic material is always present, whether the cell is alive or dead. The technique detects the absence or presence of known pathogens or genes. In addition, the primers used can be confused with similar DNA and with the target DNA, providing incorrect results (CANGELOSI; MESCHKE, 2014; RAJAPAKSHA et al., 2019).

5. FINAL CONSIDERATIONS

Identifying and monitoring identifying microorganisms in wastewater require specific and efficient methods capable of providing rapid results. Knowing the characteristics of the main methods available for analyzing pathogens and the chemical and physicochemical characteristics of the samples to be detected is crucial for researchers to select the most appropriate approach for each case. In this context, biosensors emerge as a promising technique, offering significant advantages in identifying, monitoring, and quantifying microorganisms, especially in emergency situations requiring rapid and on-site analysis. Choosing the ideal method can significantly improve the effectiveness of microbiological analysis, contribute to monitor wastewater quality and evaluate the possibilities for reuse.

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4.2 Carbon-dot pequi-nut in the development of immunosensor to detect pathogenic bacteria

Carbon-dot pequi-nut in the development of immunosensor to detect pathogenic bacteria

Rachel Menezes Castelo[a], Marília de Albuquerque Oliveira[b], Roselayne Ferro Furtado[c*], Bruno Peixoto de Oliveira[d], Lucas Vinicius Leite Martoni[e], Terezinha Feitosa Machado[d], Celli Rodrigues Muniz[d], Flávia Oliveira Monteiro da Silva Abreu[f], Sérgio Antônio Spinola Machado[e], Airis Maria Araújo Melo[g], Huai N. Cheng[h], Atanu Biswas[i], Carlucio Roberto Alves[a]

[a]Universidade Estadual do Ceará, Centro de Ciências, CCT, e-mail: rachel.menezes@aluno.uece.br

[b]Universidade Estadual do Ceará, Rede Nordeste de Biotecnologia - RENORBIO e-mail: mariliaaoliveira7@gmail.com

[c]Embrapa Agroindústria Tropical, Laboratório de Embalagens, e-mail: roselayne.furtado@embrapa.br

[d]Universidade Federal do Cariri, Instituto de Formação de Educadores, email: bruno.peixoto@ufca.edu.br

[e]Universidade de São Paulo, Instituto de Química de São Carlos, email: lucas.martoni@usp.br

[d]Embrapa Agroindústria Tropical, Laboratório de Microbiologia, e-mail: terezinha.machado@embrapa.br

[d]Embrapa Agroindústria Tropical, Laboratório de Microscopia Eletrônica, e-mail: celli.muniz@embrapa.br

[f]Universidade Estadual do Ceará, Laboratório de Polímeros Naturais, e-mail: flavia.monteiro@uece.br

[e]Universidade de São Paulo, Instituto de Química de São Carlos, email: sasmach@iqsc.usp.br

[g]Instituto Federal do Piauí campus Picos, Departamento de Ensino, email: airis.melo@ifpi.edu.br

[h]USD Agricultural Research Service, Southern Regional Research Center, New Orleans, LA 70124, USA, email: hncheng100@gmail.com

[i]USDA Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL 61604, USA, email: atanu.biswas@usda.gov

[a]Universidade Estadual do Ceará, Centro de Ciências, CCT, e-mail: carlucio.alves@uece.br

Abstract

In this study, an amperometric immunosensor for *Salmonella* Thyphimurium was designed using antibodies labeled with carbon dots (Cdots) from pequi almond (*Caryocar coriaceum*). Cdots were synthesized by pyrolysis and characterized by FTIR, UV/fluorescence, electrochemistry, zeta potential, and transmission electron microscopy (TEM). A particle size of 6.80 ± 2.13 nm was estimated, and the zeta potential was - 47.4 mV, indicating the preponderant presence of acidic groups, as confirmed by FTIR. The impedance evaluation of the response of biosensors assembled for live (Rct = 13.4 kΩ) and dead (Rct = 499.7 Ω) *Salmonella* in agreement with chronoamperometric analyses, which had their current values drastically reduced from -2.2 mA (live) to 0 mA (dead). An analytical curve for *Salmonella* was established with the limit of detection lower than 1 CFU/mL. This electrochemical biosensor using pequi carbon dots for antibody labeling showed promising results for detecting the pathogen. Thus, carbon dots can be used as substitutes for enzymes in labeling antibodies used in the design and production of sensors.

Keywords: Carbon dots, *Caryocar coriaceum*, Immunosensor, Pequi-nut, *Salmonella*

Introduction

Ensuring that safe and healthy food is accessible to all has been a global challenge. A major factor in food safety is to identify and prevent pathogenic contamination, such as *Salmonella*, in food products. It is known that salmonellosis can cause health risks with symptoms that can last for up to a week. [1] Thus a rapid and sensitive *Salmonella* detection in food samples is an important analysis. The bacteriological assay is considered the gold standard for

Salmonella detection, but this assay involves several steps and can take several days to complete [2]. Alternative detection methods for *Salmonella* Typhimurium have appeared in the literature, including ELISA [3], polymerase chain reaction (PCR) [4], surface-enhanced Raman scattering (SERS) [5], and graphene electrochemical immunosensor [6]. However, these methods often have drawbacks, such as time-consuming procedures, expensive equipment, and adequate training of technicians. The need to explore simple, rapid, cost-competitive, high-sensitivity, and specificity detection methods for pathogenic bacteria has motivated the development of electrochemical biosensors for *Salmonella*.

Electrochemical sensors appear as an effective route for replacing the previously mentioned methods, as they can detect a single chemical [7, 8] or biological molecule [9, 10]. Amperometric immunosensors have been preferred for their easy handling and ability to work with specific potential, thereby greatly reducing the interference of other chemical species [11–13].

Previous works on immunosensors for *Salmonella* detection have shown high sensitivity without the need for a sample pre-enrichment step using an enzyme-labeled antibody[14, 15]. The literature has also shown a variety of functionalization in the construction of immunosensors involving nanoparticles [16–18], some incorporating carbon dots as biomarkers, which can be switched “on-off” and highly sensitive [19], reaching limits of detection in the range of fg/mL [20].

Carbon dots are nano-sized carbon-based materials that have gained significant attention in nanotechnology and materials science. These tiny dots, typically ranging from a few to a few dozen nanometers in size, exhibit unique optical, electronic, and chemical properties, including their easy surface functionalization, low-cost synthesis, and visible light absorption [21, 22]. Recently, pequi carbon dot was developed and tested for identification of bacteria by flow cytometry [23]. This kind of carbon dot in electrochemical biosensors is being reported for the first time in this work.

In particular, an electrochemical immunosensor functionalized with nanoparticles synthesized from pequi nut can be advantageous due to possibility of adding value to this agricultural product and because it provides facile electron transfer paths and has adequate conductivity characteristics [24]. These features open the possibility of developing sensitive devices with detection limits equivalent to or lower than those needed for identifying Salmonella or similar pathogens [6, 11, 25]. In this work, carbon dot was used as a marker of secondary antibody in

the analytical response of an immunosensor for Salmonella detection. This procedure is necessary for amperometric analysis given that the antigen-antibody interaction does not involve redox reactions to have measurable current variations correlating to analyte concentration.

Materials and methods

Preparation and characterization of Carbon dots

Pequi fruits were purchased from a local market in Fortaleza. The pequi almonds were first separated manually from the fruit. Carbon dots synthesis from pequi almonds followed the bottom-up approach through the microwave pyrolysis. 2g pequi almond was macerated with H_2PO_4 (40%) and heated in the microwave for 1.5 minutes at 600 W. Then, 20 mL of ethanol and 1 mL of 2M NaOH were added, filtered on a filter paper, and centrifuged at 11,000 rpm for 20 min. The supernatant was discarded, and the precipitate dialyzed in distilled water for 48 hours. The dialyzed sample was filtered using a 22-μm pore filter and then lyophilized. For the quantitative determination of the chemical elements C, H, N, and S present in a sample, a ThermoScientific Elemental Analyzer (model FlashSmart) was used, with external calibration involving methionine, BBOT, sulfanilamide and cystine as calibration standards.

The synthesized material was characterized by Fourier transform infrared spectroscopy (FTIR) using an attenuated total reflectance detector in the wavelength range of 400-4000 cm^{-1} and resolution of 4 cm^{-1} on an FTLA 2000-102-ABB- BOMEN spectrometer (ABB Group, Quebec, Canada).

The size analysis was performed using a transmission electron microscope (TEM), model Vega 3, made by Tescan (Brno, Czech Republic). The sample was diluted 1:50 v/v, gridded for 3 min, and stained with phosphotungstic acid for 3 min. The size calculation was achieved by imageJ software.

UV-Vis analyses were performed using a Shimadzu UV-2600i UV-visible spectrophotometer, and the measured wavelength range was 190 - 800 nm.

Fluorescence analysis was carried out on a Shimadzu RF-6000 spectrofluorimeter with excitation frequency at 200 - 400 nm, and emission in the range of 400 to 800 nm, with a scanning speed of 6000 nm/s and excitation and emission slits of 5.0 nm.

The quantum yield (QY), a parameter for comparing the photoluminescence of Cdots, was calculated by the ratio between the number of photons absorbed and the number of photons emitted by a material through equation 1.

$$QY = QYs\left(\frac{I}{I_s}\right)\left(\frac{A_s}{A}\right)\left(\frac{n}{n_s}\right)^2 \quad (1)$$

where I is the integrated emission intensity, A is the absorption intensity at the excitation wavelength, n is the refractive index of the solvent, and the subscript s is related to the value of the standard fluorescent molecule.

Antibody purification and carbon dot-labeled antibodies

Polyvalent anti-Salmonella serum Poli A-I and Vi purchased from Difco® were purified by precipitation with $(NH_4)_2SO_4$ with 45% saturation. The serum was reconstituted in 3 mL of NaCl (0.85%) followed by the addition of 1.662g of $(NH_4)_2SO_4$. The obtained solution was kept under stirring for 30 minutes, refrigerated for 24h, and subsequently centrifuged at 10,000 rpm (15,303 x g) for 30 minutes at 4°C. The precipitate was dialyzed against PBS solution (10 mM pH 7.4) for 24h. After dialysis, its concentration was determined in a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Cdot-labeled antibodies were preparated from Cdot dispersed in PBS (10 mM) buffer solution (pH 7.4) with the antibody using the proportion 1:6 (antibody: Cdot, w/w).

Bacterial culture

The strain *Salmonella* enterica serovar Typhimurium (ATCC® 51812) was cultivated in BHI at 37 °C for 24 h. The resulting bacterial dispersion of unknown concentration was centrifuged for 30 min at 5000 rpm, and the supernatant was discarded. The pellet was washed with sterile 10 mM PBS (pH 7.4) and then used to prepare a suspension in which bacterial concentration was adjusted using the McFarland turbidimetric method. The standard bacteria suspensions for the calibration curve were prepared by successive dilution. The concentrations of standard dispersions were confirmed using the plate counting method from 10^8 to 10^5 CFU mL^{-1} bacteria suspension. The standard dispersions were stored at 4 °C until use, and all the experiments were done in triplicate.

Biosensor preparation

The assembly of the immunosensor was performed on the surface of a disposable gold electrode, using the self-assembled monolayers (SAMs) technique, according to the method given by Melo et al. [15] with adaptations (Fig. 1).

Screen-printed electrodes Dropsense (C220AT®) were immersed in 10 mM cysteamine ethanolic solution (cys) for 3 h. Then, the electrode was immersed in a solution of protein A (protA) 7.5 mg mL^{-1} of *Staphylococcus aureus* containing N-hydroxysuccinimide/N-(3-dimethylamino propyl) -N'-ethylcarbodiimide (EDC/NHS) (2 mM/ 5 mM) for 1 h. Before immersion, the NHS/EDC solution was kept for 30 min at room temperature; then, the prot A was added to react for one hour. The solution was left for one hour, and the electrode was immersed in the solution for 1 h, called modified cys-protA electrode. The electrode was washed with 10 mM phosphate buffer (PBS) (pH 7.4). The modified electrode was then immersed overnight in a solution of anti-*Salmonella* (AS) (2 mg mL^{-1}) under refrigeration. The non-specific binding was blocked with 1% bovine serum albumin solution (w/v) for 1 h. Finally, the modified electrode was immersed in an anti-*Salmonella*-Cdot solution in a ratio of 1:6 (w/w/)for 1 h.

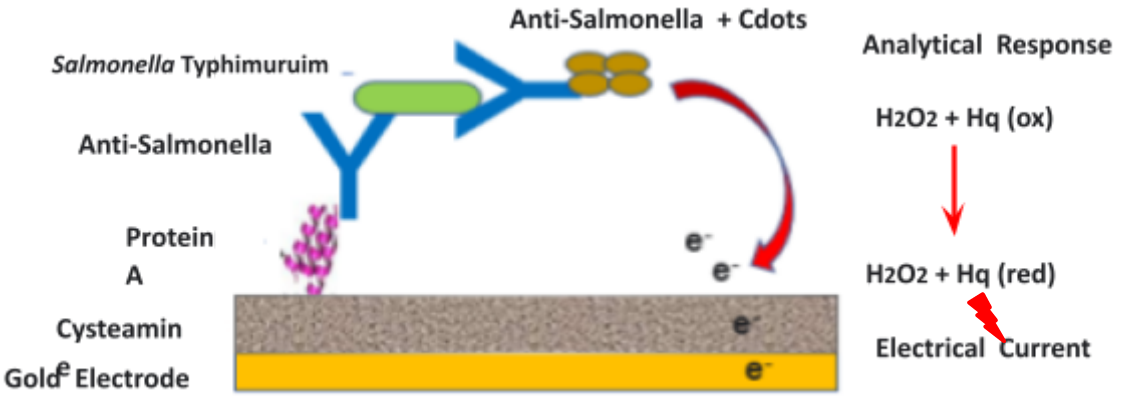


Fig. 1 Immunosensor assembly based on anti-Salmonella-Cdot and the analytical response (ox) oxidation and (red) reduction

Electrochemical measurements were made using Autolab/PGSTAT12 potentiostat/galvanostat (Ecochemie, Netherlands) coupled to a computer and controlled by NOVA 2.1.3 software (Ecochemie, Netherlands (Metrohm, Switzerland)). Chronoamperometry studies were performed at a constant potential of 0.4 mV for 120s. ANOVA [24] was used to analyze the standard curve for *Salmonella* concentrations. The limit of detection (LOD) was calculated using Equation 2.

$$LOD = Y_B + 3.3S_B \qquad (2)$$

Where Y_B is the linear coefficient, and S_B is the standard deviation of the blank [26].

Electrochemical impedance spectroscopy (EIS) data were acquired with a PGSTAT 302 potentiostat/galvanostat (Ecochemie, Netherlands) system, controlled by FRA2 software (Metrohm, Switzerland). EIS experiments were performed, in 0.1 mol L^{-1} KCl solution containing 5.0 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6$, under the open circuit potential (OCP), in the frequency range between 0.1 Hz and 100 kHz with an amplitude of 10 mV and under the open circuit potential (OCP) conditions in 0.1 mol L^{-1} KCl solution containing 5.0 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The light source was an UV LED (9 W) operated by a lab-made controller board (UNO) integrated into a relay and programmed by Arduino software.

Statistical analyzes

Three replications were performed in the assays and used to obtain mean and standard deviation results. The linearity of the calibration curve was analysed analysis of variance using Origin 8.0.

Results and discussion

Carbon dots characterization

The synthesis of Cdots from the pequi almond showed a reaction yield of 5%. Its quantum yield, calculated from fluorescence and UV-Vis spectra, was 9% (Fig. 2b). Since the elemental analysis indicated a nitrogen content of 11% and a carbon/nitrogen ratio of 1/3, nitrogen in Cdot significantly affected the quantum yield value. From a comparison of the Cdots obtained from the pequi almond with other Cdots obtained from natural sources, this QY value was higher than or compatible with the quantum yield of Cdots synthesized from *Cissus quadrangularis* leaf, which was above 5% [27], cellulose fibers was of 1% [28], and quantum dots extracted from fig leaves 3% QY [29]. According to fluorescence spectroscopy (Fig. 2a) increased the luminescent intensity of the Cdots. It shifted the emission maximum to a region closer to red, known as the bathochromic effect [30]. This excitation wavelength-dependent photoluminescence behavior is characteristic of Cdots based on natural sources [31, 32] and may be linked to the nitrogen content of the Cdots sample [33, 34].

The maximum emission peak occurred at a wavelength of 360 nm, representing an average fluorescence intensity of 23,314 a.u. From 290 nm onwards, there was a decrease in luminescent intensity, a fact that may be related to quantum confinement, size distribution, or the presence of emitting traps on the Cdot's surface [35]. Groups on the Cdots surface,

especially those containing oxygen, nitrogen, and hydrogen atoms, can have different energy levels that make the emission dependent on the excitation energy. In addition, the fluorescence emission can change due to particles of different sizes and emissive sites' distribution on its surface [36].

The UV-vis absorption spectrum (Fig. 2b) showed maximum absorption in the UV region, which decreased in the visible region. Cdots have characteristic peaks (UV) at 253 and 280 nm in the π - π^* electronic transition region corresponding to sp^2 and n - π^* carbon bonds of surface carbons with structures containing nitrogen and/or oxygen [37], agreeing with the emission spectrum at 300 and 360 nm, indicating that the blue band in the photoluminescence results from transitions in the Cdots core and defect surface with sp^3 hybridization, respectively [38].

The infrared spectrum (Fig. 2c) showed intense -OH bands at 3400 cm^{-1}. The presence of C=O bonds of the carboxylate ion and C=C of the carbon structure was confirmed by absorptions at 1654 and 1391 cm^{-1}, respectively. Another high-intensity band was observed at 2930 cm^{-1}, which can be attributed to the asymmetric stretching of C-H bonds in the aliphatic portion of the Cdot's surface. The collapsed band can characterize the amino groups at 3200 cm^{-1} and the region with several peaks at 600-500 cm^{-1}, corresponding to the N-H bonds of primary amine. [23].

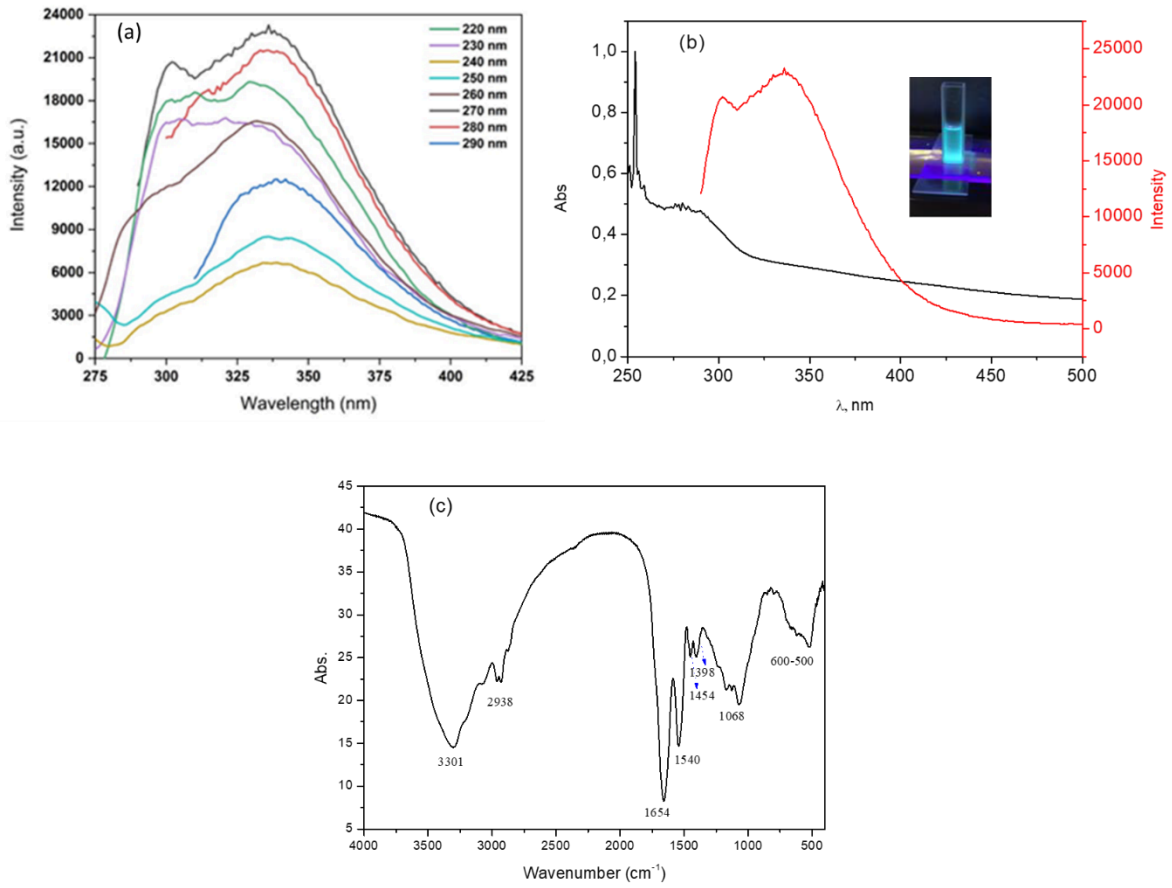


Fig. 2 (a) Fluorescence emission spectra of Cdots; (b) UV-Vis and fluorescence spectra at λ = 270nm; (c) FT-IR spectrum of Cdots

Through transmission electron microscopy analysis (Fig. 3a), the Cdots were found to have a spherical shape in the regions where they appeared dispersed. The measured particle average size was 6.80 ± 2.13 nm and had unimodal behavior (Fig. 3c and 3d). The size and form of the Cdots are in agreement with other studies [39, 40]. The zeta potential of Cdots in aqueous media was -47.4 ± 0.3 mV, suggesting the preponderant presence of carboxylic acid groups on the surface, and other oxygenated functional groups [41] (Fig. 3b). The highly negative value of the zeta potential means that the electrostatic forces are predominantly repulsive, indicating that the system has a high stability against aggregation. These results agree with the TEM image, where Cdots were observed to be well dispersed, in agreement with the earlier findings of [42].

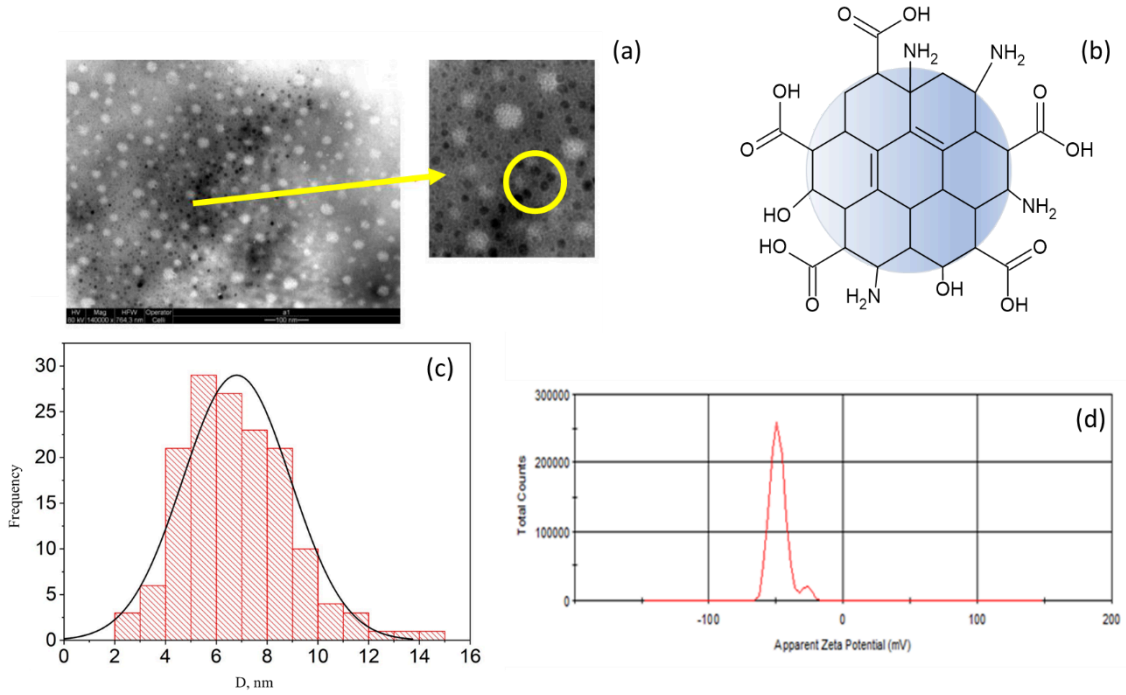


Fig. 3 (a) Transmission electron micrograph of carbon dots diluted in water at a ratio of 1:50 v/v, deposited on a grid for 3 min and stained with phosphotungstic acid for 3 min; (b) probable structure of the carbon dot with carboxylic acid groups around it; (c) statistical particle size distribution; (d) distribution graph of the zeta potential of the carbon dots solution in water

Performance and characterization of the biosensor

Chronoamperometry analysis

The chronoamperometric behavior (Fig. 4a) of the biosensor assembled and tested with active and inactive Salmonella showed a drop in the electrical signal from -2.2 mA to zero. This observation is reasonable, as Salmonella is a filamentous bacterium that, when alive, has its flagella always in movement, capable of interacting with the antibody. After slow pasteurization, the bacteria cannot interact with the antibody and may precipitate out of the medium; thus, no response is observed. Biosensor performance was evaluated on samples with different concentrations of Salmonella, simulating a contaminated sample at concentrations of 1 - 10^7 CFU.mL^{-1}. The mass ratio of Cdots and antibody for the assembly of the immunosensor in the proportion of 6:1 (m/m) made it possible to visualize the decrease in the electrical signal with the increase in the concentration of Salmonella. Thus, it was possible to construct an analytical curve with an amperometric response of the immunosensor at different concentrations of *S*. Typhimurium, thereby establishing a linear relationship between the current signal (A) and the analyte concentration (CFU.mL^{-1}) (Fig. 4b).

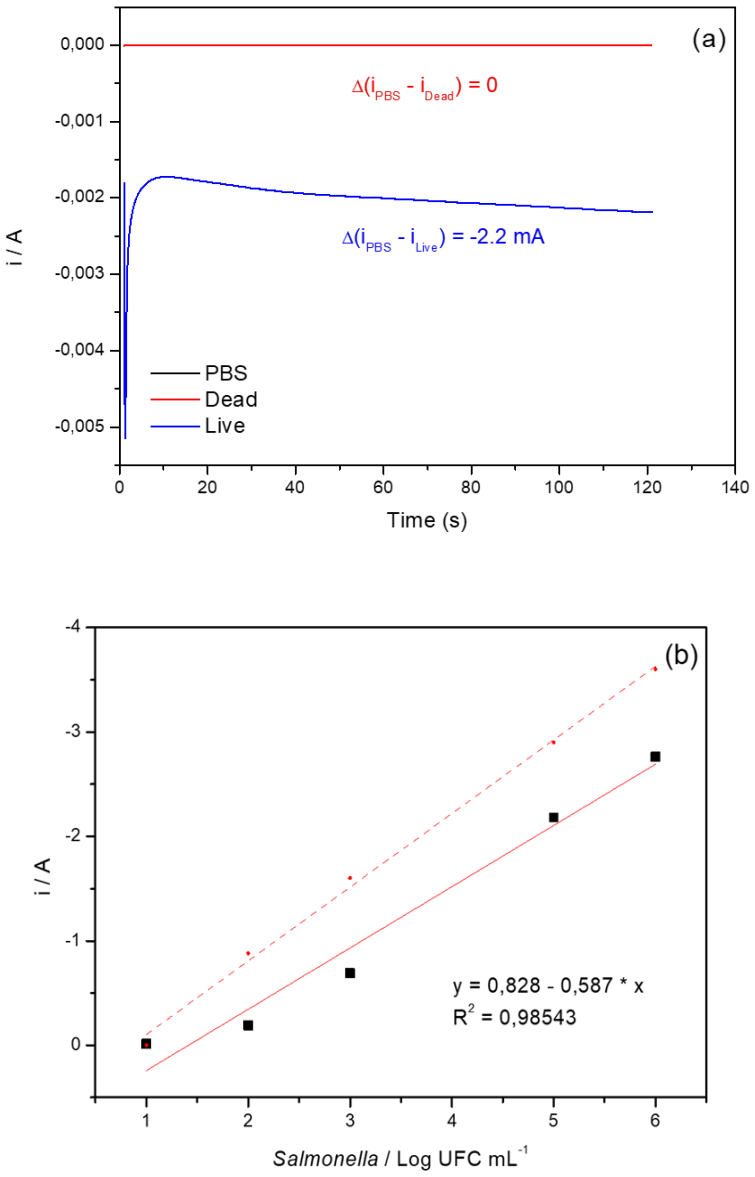


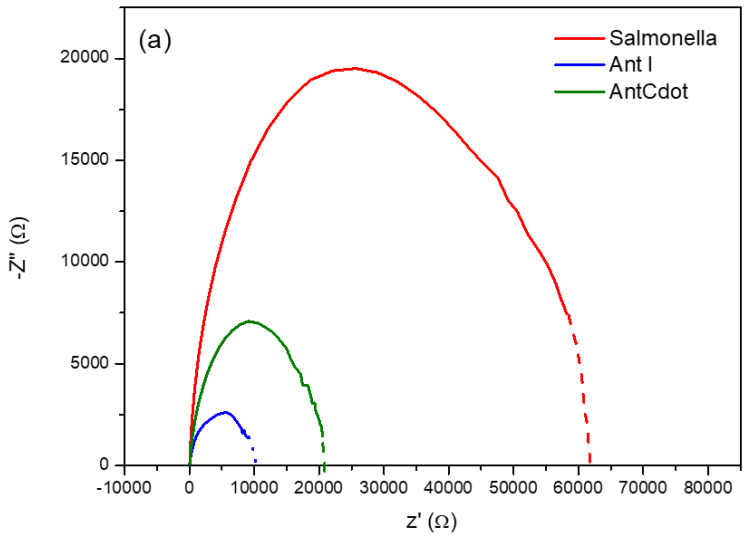
Fig. 4 (a) Amperometric response for biosensors in live and dead Salmonella. The chronoamperogram of the inactive salmonella biosensor coincided with the PBS curve, providing zero difference; (b) Analytical curve for immunosensor assembled with Cdots-labeled antibody at m/m ratio of 1/6

The immunosensor was able to distinguish between different concentrations of Salmonella ($p < 0.05$). ANOVA[43] confirmed its linearity, so a quantitative behavior of the biosensor for the pathogen could be confirmed. The LOD of the device was < 1 CFU/mL, indicating a good sensitivity of the device, compared to biosensors assembled with the peroxidase enzyme with LOD 10 CFU/mL [15] and compared to syringe actuation biosensors using nuclear membrane filtration and nanozyme signal amplification, which despite of the simplest assembly obtained a LOD of 12 CFU/mL [44]. Similar behavior was seen for a nanocluster colorimetric

aptasensor in the detection of *S.* Thiphymurium in eggs that exhibited a broad linear response in the concentration range of 10^1–10^6 CFU/mL with a detection limit as low as 1 CFU/mL [45].

Electrochemical Impedance Spectroscopy (EIS) analysis

The manufacturing process of the electrochemical biosensor was elucidated by EIS in 5.0 mM $[Fe(CN)_6]^{3-/4-}$. After assembling the biosensor with live Salmonella at a concentration of 1 CFU/mL and with dead Salmonella previously pasteurized at 65ºC for 30 min.[46, 47], the impedimetric data for the two situations were collected and are shown in Figure 5. Figure 5a shows the Rct measurements for anti-Salmonella (Rct = 10.4 KΩ), Salmonella (Rct = 61.7 KΩ), and the last step with ant-Salmonella-Cdot (Rct = 20.5 KΩ). As the electrode surface was modified, the Rct did not increase in an orderly fashion due to the non-conducting properties of these protein structures as well as the small size of molecules in each assembly step, e.g., cysteamine [48, 49]. However, a trend is observed in which there is an increase in resistance in Salmonella at low frequency and at the end of the assembly in ant-Salmonella-Cdot, where the resistance drops to 20.5 KΩ, giving evidence that Cdot has semiconductor properties. Various types of carbon (and quantum) dots with semiconductor characteristics have become potential new platforms for application as fluorescent probes [50]. For this system, the earlier literature reported that the value of quantum yield is directly related to charge transfer, as high quantum yield offers greater resistance to charge transfer because it has fewer electrons in the excited state to contribute to conductivity [51]



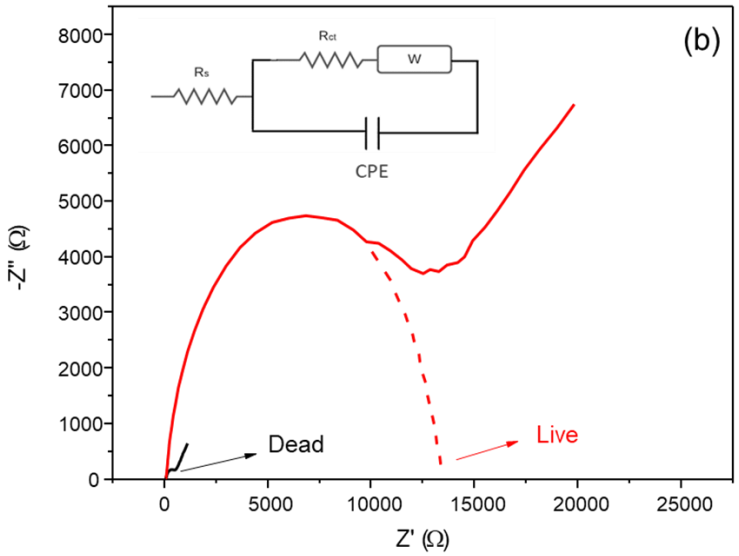


Fig. 5 (a) The typical Nyquist diagram for the biosensor formation steps; (b) Nyquist diagram for biosensors with live and dead Salmonella with a Randles circuit, for live only, comprising the uncompensated electrolyte resistance (Rs), in series with the dielectric layer capacitance (CPE), transfer resistance load (Rct) and Warburg impedance (W), in circuit form

Additionally, unlike cysteamine, the BSA immobilization step (used as a blocking molecule for non-specific antibody sites during biosensor assembly) caused a significant increase in charge transfer resistance (curve not shown - Rct = 74.0 kΩ). This high resistance to electrical conductivity is due to the lack of electrons available in the excited state, and the protein forms a blocking barrier for the diffusion process on the modified electrode surface[52]. Thus, the protein layer is considered a porous insulating layer and can work like a capacitive element. Furthermore, the high protein adsorption density on the electrode surface leads to a small interface area (Rs), so the solution resistance within the protein layer interface is not negligible (Huang et al., 2019).

Figure 5b shows that the resistance of the biosensor for dead Salmonella (Rct = 499.7 Ω) and gives a lower Rct value than the live bacteria (Rct = 13.4 – 20.5 KΩ in Fig. 5b, which it gives the Nyquist diagram in the Randles circuit for the biosensor assembled with live Salmonella). The dead Salmonella shows that the bond between the pathogen and the antibody conjugated to Cdots there is not, thereby interrupting the passage of electrons. This behavior allows the device to avoid false-negative results that can be generated by the PCR technique, where its detection mechanism by DNA may cause a false-positive response even to dead bacteria [53]. Therefore, the biosensor described in this work becomes extremely important for industrial

processes for monitoring the food quality, as it would not invalidate an entire batch of food materials because it differentiates between living and dead microorganisms [54].

Conclusions

Carbon dot technology represents an exciting new opportunity for innovation involving biobased materials. This article used antibodies labeled with Cdots from pequi almond to build a biosensor for Salmonella detection. The synthetic route to obtaining Cdots from pequi almonds was simple and quick, with a process yield of 5%. The size of the Cdots was 6.8 nm, compatible with the range determined for materials of this nature, with a spherical appearance and good dispersion. The quantum yield of 9% was satisfactory when compared to other similar materials in the literature. The immunosensor developed with pequi Cdots could distinguish between different concentrations of live Salmonella ($p < 0.05$) if quantitative behavior for the pathogen was assumed. The LOD calculated for this device was ten times more sensitive relative to biosensors developed with the peroxidase enzyme, which places the biosensor in this work on the list of ultrasensitive devices. Another important aspect is that this device distinguishes active and inactive Salmonella, and this is an advantage over other detection methods, like PCR analysis.

Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4.3 Rapid and sensitive detection of *Salmonella* Typhimurium in solid and liquid food matrices by an amperometric biosensor

Rapid and sensitive detection of *Salmonella* Typhimurium in solid and liquid food matrices by an amperometric biosensor

[a]Rachel Menezes Castelo, [b]Marília de Albuquerque Oliveira, [c]Denilson Oliveira Menezes, [d]Airis Maria Araújo Melo, *[e]Roselayne Ferro Furtado, [f]Terezinha Machado Feitosa, [g]Carlucio Roberto Alves

* Corresponding author: Roselayne Ferro Furtado

e-mail: roselayne.furtado@embrapa.br

[a]Universidade Estadual do Ceará, Centro de Ciências, CCT, Fortaleza, Ceará, Brazil

[b]Universidade Estadual do Ceará, Rede Nordeste de Biotecnologia – RENORBIO, Fortaleza, Ceará, Brazil

[c]Universidade Estadual do Ceará, Departamento de Química, Centro de Ciências, Fortaleza, Ceará, Brazil

[d]Instituto Federal de Educação, Ciência e Tecnologia do Piauí, *Campus* Picos, Picos, Piauí, Brazil

*[e]Embrapa Agroindústria Tropical, Laboratório de Embalagens, Fortaleza, Ceará, Brazil,

[f]Embrapa Agroindústria Tropical, Laboratório de Microbiologia, Fortaleza, Ceará, Brazil

[g]Universidade Estadual do Ceará, Departamento de Química, Fortaleza, Ceará, Brazil, https://orcid.org/0000-0001-7164-7467

Abstract

The frequency and severity of outbreaks caused by *Salmonella* in food and water have encouraged the advancement of rapid and sensitive methods for pathogenic microorganism detection. The amperometric biosensor functionalized with Pequi quantum dots is an option for a rapid quantitative detection of *Salmonella* in liquid and solid food matrices. The biosensor demonstrated excellent specificity and was effective in detecting *Salmonella* in food with a very low limit of detection (LOD) below 1 CFU/mL. This study highlights the potential use of the amperometric biosensor with Pequi- carbon dot-conjugated secondary antibody for a rapid and sensitive detection of *Salmonella* in various food samples.

Key words: Immunosensor, Pequi-nut, Carbon dots, *Salmonella*, food waste water, cheese.

Introduction

Food contamination is the main cause of Salmonellosis, such as eggs, chicken, pork and their products, dairy products, and vegetables (Galán-Relaño et al. 2023; Mkangara 2023). Therefore, rapid and specific detection of *Salmonella* in food is essential to ensure food safety and reduce the risk of bacterial threats to humans. Currently, *Salmonella* detection is mainly divided realized by conventional and rapid methods. The conventional method is usually based on cultivation, which it is accurate and reliable but time-consuming and laborious (Teklemariam et al. 2023). Rapid detection methods mainly include molecular, immunological, and biosensor methods, such as polymerase chain reaction (PCR) (Tabatabaei et al. 2021), enzyme-linked immunosorbent assay (ELISA) (Zhao et al. 2021), and surface plasmon resonance (SPR) (Jebelli et al. 2020). These methods are specific and sensitive but have some disadvantages. For example, molecular methods require expertise in genomics lower other related knowledge, and these often require long pre-enrichment steps to achieve detection limits (Ding et al. 2024). Furthermore, some of these methods need to be coupled with biorecognition molecules to effectively capture target strains. Thus, biomolecules play a particularly important role in ensuring the specificity, stability, and accuracy of the assay, as well as its cost, and further research is needed (Ding et al. 2024).

Electrochemical immunosensors have gained prominence in research involving the identification of pathogenic microorganisms due to their high sensitivity, selectivity, and low limit of detection (LOD). The main advantages of electrochemical sensors are high accuracy and repeatability, their low detection limits, and their ability to provide real-time measurements (Umapathi et al. 2022). Many biosensors for *Salmonella* sp. have been developed and reported (Liu et al. 2024; Muhsin et al. 2022), but not many studies evaluate biosensor performance in real samples.

In addition to increasingly specific devices, nanoparticles have been used to increase the electrical signal and identify increasingly specific target molecules. Carbon quantum dots (CDs) are a new type of carbon-based nanostructured material that exhibits fluorescence and specific characteristics. They are unique quasi-spherical carbon nanoparticles with ultrafine particles measuring less than 10 nm (Barrientos et al. 2023). Typically composed of a carbogenic core, with functional groups attached to surface sites, which increase the possibility of chemical interaction with other molecules. Carbon dot can substitute enzymes and other molecules in the use as markers. In this work, carbon dot-conjugated secondary

antibodies were evaluated in the response of a amperometric biosensor which detected efficiently *Salmonella* using enzymes – conjugated secondary antibodies (Melo et al. 2021a).

Carbon dots synthesis some papers about carbon dots synthesis by fruit but its reports some about other fruits such as carbon dot synthesis by lemon via green hydrothermal method (Slewa 2024). Carbon quantum dot prepared by hydrothermal procedure using a green biomass resource, Morinda coreia fruits, obtained spherical carbon dots with a mean diameter of 1.99 nm, they possessed functional groups like carboxyl and amide on their surface and emit light of wavelength of 490 nm under 390 nm excitation. The fluorescent Morinda coreia carbon dots shown 17% quantum yield (Tony Elizabeth et al. 2024). Chandra et al. synthesized fluorescent carbon quantum dots from Jatropha fruit by using a one-pot hydrothermal approach, for the first time. CDs exhibited bright blue fluorescence emission with a high quantum yield of 13.7 % that have been used as a fluorometric sensor for the detection of pesticides (Chandra et al. 2022).

The use of the Pequi almond (*Caryocar cariaceum*) is justified by the fact that it is a fruit widely found in the Brazilian cerrado and semi-arid region, the extraction of the pequi pulp generates a residue (stone) that has an almond inside that is little commercially exploited. Thus, the almond is of economic interest due to its low cost. The literature has shown a few papers about pequi fruit. However, due the biotechnological potential of quantum dots from Pequi almonds, this study aimed to develop and evaluate the analytical potential of the electrochemical immunosensor for *Salmonella* sp. in different matrices such chicken washing water and cheese due they are a food that is very easy to contaminate throughout its production and storage process.

Experimental

Pre-treatment of samples of different food matrices

The analyses were performed according to standard protocols for the identification of *Salmonella* Typhimurium (ATCC® 51812), *Staphylococcus aureus* (ATCC® 12600) and *Escherichia coli* (ATCC® 4157). The coliform analysis followed the most probable number (MPN) methodology, while the *Salmonella* test involved growth on selective agar medium up to the biochemical series (Silva et al. 2021).

Samples of wastewater from egg and chicken washing were collected from a commercial establishment in the local region (Fortaleza, Brazil). The impurities suspended in the water were filtered with Whatman qualitative filter paper, diam. 25 mm and the wastewater were enriched with different concentrations of *Salmonella* Typhimurium ATCC® 51812 (10^1 to 10^5 CFU mL^{-1}); these solutions were used to construct a standard curve. In addition, the *Salmonella* concentration was determined in non-sterile lake water samples.

Samples of curd cheese sold in Fortaleza-Ceará (25 g of Coalho cheese in 50 mL of 10 mM PBS pH 7.4) were macerated in Stomaker®400 at 300 rpm for 10 minutes. The material was centrifuged twice at 10,000 rpm, 4°C for 30 minutes and filtered with Whatman qualitative filter paper, diam. 25 mm to remove fat and protein from the sample.

Production carbon-dot from pequi

Pequi fruits were purchased from a local market in Fortaleza. The pequi almonds were first separated manually from the fruit. Carbon dots synthesis from pequi almonds followed the bottom-up approach through the microwave pyrolysis method. An amount of 2g pequi almond was macerated together with H_2PO_4 (40%) and heated in the microwave for 1.5 minutes at 600 W. Then, 20 mL of ethanol and 1 mL of 2M NaOH were added, filtered on a filter paper, and centrifuged at 11,000 rpm for 20 min. The supernatant was discarded, and the precipitate dialyzed in distilled water for 48 hours. The dialyzed sample was filtered using a 22-µm pore filter and then lyophilized.

Assembly of the immunosensor on a disposable electrode

The assembly of the immunosensor was performed on the surface of a disposable gold electrode, using the self-assembled monolayers (SAMs) technique, according to Melo et al. (2021) with adaptations. Screen-printed electrodes Dropsense (C220AT®) were immersed in 10 mM cysteamine ethanolic solution (cys) for 3 h. Then, the electrode was immersed in a solution of protein A (protA) 7.5 mg mL^{-1} of *Staphylococcus aureus* containing N-hydroxysuccinimide/N- (3-dimethylamino propyl) -N'-ethylcarbodiimide (EDC/NHS) (2 mM/ 5 mM) for 1 h. Before immersion, the NHS/EDC solution was kept for 30 minutes at room temperature and, then the prot A was added to react for one hour. In the sequence, the electrode was immersed in the solution for 1 h, called modified cys-protA electrode. After that, the electrode was washed with 10 mM phosphate buffer (PBS) (pH 7.4). The modified electrode was then immersed overnight in a solution of anti-*Salmonella* (AS) (2 mg mL^{-1}) under refrigerated condition. The non-specific binding was blocked with 1% bovine serum

albumin (BSA) solution (w/v) for 1 h, and finally the modified electrode was immersed in an anti-*Salmonella*-Cdot solution (1:6 w/w) for 1 h.

Electrochemical characterization

Electrochemical measurements were realized using Autolab/PGSTAT12 potentiostat/galvanostat coupled to a computer and controlled by NOVA 2.1.3 software (Ecochemie, Netherlands). Chronoamperometry studies were performed at a constant potential of 0.4 mV for 120s. All measurements were performed at room temperature in a 10 mL voltammetric cell PBS 10 mM solution (pH 7.4) containing 600 mM H_2O_2 and 4 mM hydroquinone. PBS solutions (control) werewere measurements for comparison purposes.

. The potential of the gold electrode was -75 mV for 120 s.

Immunosensor Performance

Calibration curve

S. Typhimurium dilutions (10^1 to 10^5 CFU mL^{-1} in 0.1 mol L^{-1} PBS, pH 7.4) were tested to cover a wide range of concentrations in the construction of the calibration curve. The analytical response was obtained by immersing the immunosensor in 100 μL of each *Salmonella* dilution for 1 h, and PBS buffer was used as the control. Each measurement was evaluated in triplicate. From the calibration curve, the following performance parameters were obtained: detection limit, detection time, linearity coefficient, and linearity range.

Precision

Precision was calculated based on the repeatability of the results and was obtained by analyzing the relative standard deviation (RSD) based on Eq. (1).

$$RSD = \frac{Standard\ deviation}{average} * 100$$

LOD

The detection limit (LOD) of the device was determined from the analytical curve obtained through the equation of the straight line through Eq. (2), considering that Y_B is the linear coefficient of the analytical curve and S_B is the standard deviation of the blank (INMETRO 2018).

$$LOD = y_B + 3S_B$$

Cross-reactivity Assessment

To examine the cross-reactivity of the amperometric immunosensor, two different bacterial strains, *Escherichia coli* (ATCC® 10536) and *Staphylococcus aureus* (ATCC® 12600) were used in the assays. The bacterial inoculum was prepared with a dilution of 10^1 CFU mL^{-1} in 0.1 mol L^{-1} PBS at pH 7.4 for each bacterium. In the mixed bacterial inoculum, equal proportions of each bacterial dilution were homogenized, maintaining the concentration of 10^1 CFU mL^{-1} in the inoculum mixture. The analytical response was obtained accordingly by chronoamperometry by polarizing the gold electrode at -75 mV until reaching a stable baseline in 120 s.

Tests on food waste water and cheese

Four real samples were analyzed: wastewater used for washing farm chicken (WWC), wastewater used for washing farm eggs (WWE) and two brands of Coalho cheese (CC1 and CC2) sold in large supermarkets. The samples were previously submitted to microbiological analysis and inoculated with *S.* Typhimurium at a low level (10^1 CFU mL^{-1}). The response was obtained by chronoamperometry under the above-mentioned conditions.

Statistical analysis

Three replications were performed in the assays and used to obtain mean and standard deviation results. The linearity was confirmed by analysis of variance using Origin 8.0.

Results and discussion

The biosensor assembly with the antibody labeling by carbon dots was successfully obtained for the identification of *Salmonella* in PBS buffer 10mM pH = 7.4. From this, other tests were carried out to verify the biosensor's ability to identify *Salmonella* in other food matrices. The cheese was chosen for its high ease of contamination by bacteria, throughout the manufacturing process until its storage (Oliveira et al. 2021). Therefore, the microbiological analysis was performed for cheese samples from this study as described in Table 1. For *Salmonella* analysis, absence or presenceis enough since the Brazilian legislation only allows consumption if the result of the analysis is absent (BRASIL, 2022).

Table 1 Microbiological analysis of chicken washing wastewater (WWC), egg washing wastewater and Coalho cheese (CC) samples. The results were obtained after 5 days.

Samples	*Salmonella*	*E. coli* (NMP/mL)	*S. aureus* (UFC/mL)
WWC	Absent	1,100	2.0×10^3
WWE	Absent	9.2	< 100
CC1	Absent	23	< 100
CC2	Absent	16	2.0×10^3

The conventional analysis method confirmed the absence of *Salmonella* in the samples (Table 1). The same method was used to verified to accuracy of the biosensor. The samples not presented colonies for *Salmonella*. Brazilian and other international regulamentation establishes microbiological standards for food and determines that food suitable for human consumption must present the absence of *Salmonella* sp. in 25 g of the sample (BRASIL, 2022). There was a prevalence of *Staphylococcus aureus* for the cheese samples and the concentration of *E. coli* was insignificant, according to food safety criteria (Minas Gerais, 2021), since high counts of total coliforms can result from inadequate hygiene practices (Franceschi et al. 2020) or contamination of environmental water (Metz et al. 2020).

When samples of washing water from chicken slaughterhouses were evaluated, the absence of *Salmonella* was observed (Table 1), indicating that the establishment has been disposing of water properly. The investigation of the presence of *Escherichia coli* and *Staphylococcus aureus* were within the concentration range permitted by ANVISA. The absence of *Salmonella* allowed the use of the samples, simulating contamination, to quantify the pathogen in the concentration range of 10^1 to 10^7 CFU/mL and thus validate the operation of the biosensor in different matrices.

Immunosensor performance parameters

Calibration curve

The amperometric immunosensor had its electric current measured from the electronic transfer of oxidation-reduction reactions involving hydrogen peroxidase in a PBS solution in the presence of peroxide/hydroquinone (Pimenta-Martins et al. 2012). The chronoamperometric response of the immunosensor in the presence of concentrations of 10^1 to 10^5 of *S.* Typhimurium in water and cheese is shown in Fig. 1.

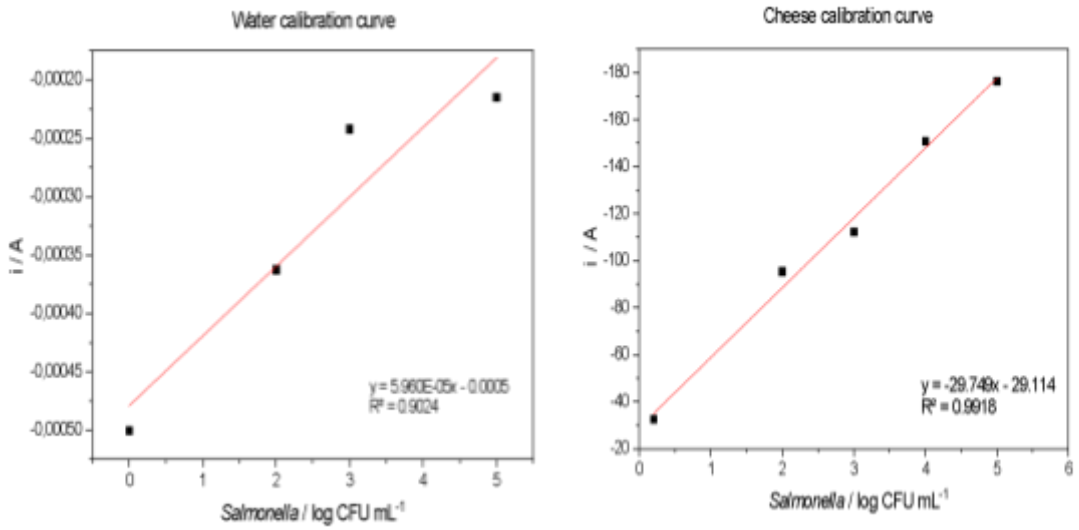

Fig. 1 The chronoamperometric response of the immunosensor in the presence of concentrations of 10^1 to 10^5 of *S.* Typhimurium in water and cheese samples

The immunosensor was able to clearly distinguish the control (PBS) from the *Salmonella* dilutions with ($p < 0.05$). It was possible to verify in the calibration curve a linear trend from 10^1 to 10^5 CFU mL^{-1}. At concentrations greater than 10^6 CFU mL^{-1}, a loss of sensitivity was observed, indicating a system saturation.

The detection time was 2h, including the incubation time for antigen recognition, the time for binding of the antibody labeled with CDs and the time for amperometric measurement. This successful result demonstrates the potential use of the biosensor to be used as a promising method for *Salmonella* detection in the food industry, especially for the rapid screening of contaminated samples.

Furthermore, the CD-labeled antibody showed satisfactory performance. Due to their excellent electron transport capacity, these nanoparticles are frequently used in electrochemical biosensors, and the signals (i.e., current) are altered due to the interaction with biomolecules, facilitating the detection and identification of target analytes (Garg e Prasad 2023; Jose et al. 2024). Nanomaterials incorporated into the working electrodes amplify the electrochemical signals under mild conditions, where nanomaterials improve performance by being used as nanocatalysts that facilitate the production of electroactive species and nanocarriers that act as redox-active species, thus improving the efficiency of the biosensor (Flores-Ramírez et al. 2024; Patel et al. 2022).

Another important parameter that complements the adequate performance of the device is the precision of the biosensor. This parameter is evaluated by the relative standard deviation (RSD). The precision of the biosensor is another significant parameter in addition to the adequate performance of the device. This parameter is evaluated by the relative standard deviation (RSD). The range of the 1–7% RSD obtained is an acceptable result, considering the large number of steps in the construction of the immunosensor. Values smaller than this study, the calculated values for repeatability and reproducibility were 4.12 % and 2.68% respectively. [11].

Immunosensor specificity

The specificity of the amperometric immunosensor was investigated against *Escherichia coli* and *Staphyloccocus aureus*. *E. coli* was chosen because it is a group phylogenetically related to the genus *Salmonella* (KNIREL et al. 2002; Melo et al. 2021b; Péterfi et al. 2007), while *S. aureus* is an opportunistic pathogen in humans (Boss et al. 2016; Melo et al. 2021b). *Salmonella* and *E. coli* are part of the group of gram-negative bacteria, which, in addition to having a peptidoglycan cell wall, have an outer layer (lipid bilayer containing lipopolysaccharides/endotoxin (LPS), lipoproteins and porins), which makes these bacteria more resistant to antibiotics. For these reasons, they are especially important for study and is important for public health. *S. aureus* is part of the gram-positive group and has structural differences between Gram-positive and Gram-negative bacteria, especially in the composition of cell walls. Gram-positive bacteria have a thick layer of peptidoglycan, while Gram-negative have a more complex cell wall, as explained previously.. These differences may influence the interaction between the antibodies immobilized in the sensor and the specific antigens of each bacterium. However, the presence of conserved antigens between different species can result in cross-reactions, affecting specificity.

In the analysis of the immunosensor against *S. aureus*, the device presented a very low electrical signal -2.7x10^{-8} μA, while for *E. coli*, which is very similar to *Salmonella* (and is even an indication of the presence of *Salmonella*) not had a higher signal (Fig. 2).

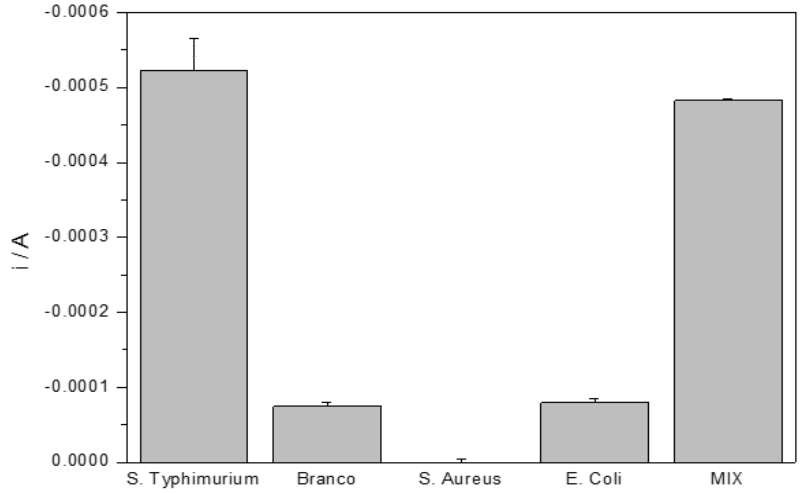

Fig. 2 Cross-reactivity test of the immunosensor for control (PBS pH 7.4), pure, and mixed cultures of *S.* Typhimurium*, S. aureus, E. coli* (10^1 CFU mL^{-1})

The biosensor was able to effectively differentiate the presence of Salmonella, minimizing cross-reactions with *E. Coli* and *S. Aureus*. The mixture of the three different cultures at concentrations of 10 CFU mL^{-1} to evaluate the recognition of Salmonella in the presence of *E. coli* and *S. aureus* was -4.83 x 10^{-4} ± 2 x 10^{-6} A in relation to the electrical signal for the biosensor with only Salmonella (-5.22 x 10^{-4} ± 2.0 x 10^{-5} A). The difference for the two conditions was not significant, and it can be stated that there was no change in the signal for these two conditions.

Assessment of the immunosensor response in the cheese and water samples

The assessment of the proposed immunosensor for detection of *S.* Typhimurium was analyzed in complex matrices such as wastewater and cheese. *S.* Typhimurium was inoculated at a concentration of 10^1 CFU/mL in wastewater from washing chicken and eggs, as well as two brands of cheese commercially (Fig. 3).

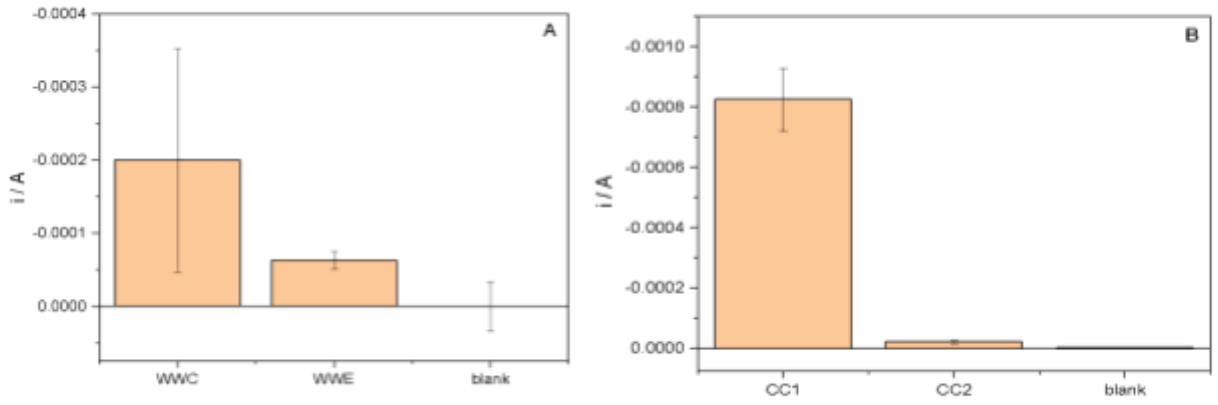

Fig. 3 Amperometric response of the immunosensor at concentrations of 10^1 of *S. Typhimurium* in wastewater (A) and cheese (B) samples. Measurements obtained in 10 mM of PBS buffer (pH 7.4) 300 mM of H_2O_2 () and 3 mM of hydroquinone (), sweep potential 75 mV for 120 s.

In the chicken washing wastewater (WWC), egg washing wastewater (WWE) and Coalho cheese (CC) samples, the immunosensor identified low concentrations of *Salmonella*. As in the CC1 and CC2 samples, even though the cheese presented a complex bacterial matrix, the device was sensitive for identifying the bacterium. In the WWC and WWE samples, the immunosensor identified concentrations of 10^{-1} CFU/mL of *Salmonella* with an electrical response of -2.2 x 10^{-4} and -6.2 x10^{-5} A, respectively. Xiang et al. (2015) developed an electrochemical immunosensor to detect *Salmonella* based on a glassy carbon electrode modified with high-density gold nanoparticles (AuNps) dispersed in chitosan hydrogel. The composite film has been used as a platform for the immobilization of biorecognition elements, such as antibody. A sandwich electrochemical immunosensor has been assembled after incubation with *Salmonella* and the horseradish peroxidase (HRP)- conjugated *Salmonella* secondary antibody. The immunosensor showed a linear range from 10 to 10^5 CFU mL^{-1} with a low detection limit of 5 CFU mL^{-1}.

The literature reports other sensors functionalized with nanomaterials, an example is an aptamer-based electrochemical sensor that had its detection ultrasensitive to bisphenol A (Xiao et al. 2020). Its sensitivity was attributed to functionalization with MWCNT/nanocomposite SiO2@Au and the current value was 25 μA for a concentration of 10 nM of bisphenol A

Conclusion

The immunosensor had a very rapid response with a low detection limit. These two characteristics are extremely important in a food outbreak scenario, where a rapid response is needed to identify the contamination cause. Furthermore, this immunosensor showed a high specificity for the detection of *Salmonella* sp. when it was tested in isolated and mixed cultures of *E. coli* and *S. aureus*. The use of the immunosensor for analysis in the cheese and waste water samples has been confirmed and is fast, easy handling, and has a low detection limit. So, it is recommended to evaluate the performance of the device in each matrix, considering the peculiarity of each one of them. The immunosensor with Pequi carbon dot has a great potential to be used as a tool for preventing and reducing outbreaks caused by *Salmonella* and can function as a basis for the development of immunosensors for the detection of other bacteria species.

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5 CONCLUSÕES

A rota sintética para obtenção de Carbon dots a partir da amêndoa de pequi foi simples e rápida, com rendimento de processo de 5%. O tamanho dos Cdots foi de 6,8 nm, compatível com a faixa determinada para materiais desta natureza, com aspecto esférico e boa dispersão. O rendimento quântico de 9% foi satisfatório quando comparado a outros materiais similares na literatura.

O imunossensor desenvolvido com Carbon dots da amêndoa do Pequi conseguiu distinguir entre diferentes concentrações de *Salmonella* viva ($p < 0,05$) caso fosse assumido comportamento quantitativo para o patógeno. O LOD calculado para este dispositivo foi dez vezes mais sensível em relação aos biossensores desenvolvidos com a enzima peroxidase, o que coloca o biossensor deste trabalho na lista dos dispositivos ultrassensíveis, destacando que o dispositivo conseguiu distinguir entre *Salmonella* viva e morta, o que é uma vantagem sobre outros métodos de detecção, como a análise PCR.

A alta especificidade para detecção *de Salmonella* sp. quando testado em culturas isoladas e mistas de *E. coli* e *S. aureus* em amostras de queijos e águas residuais foi confirmada. Sendo um dispositivo com resposta rápida, de fácil manuseio e com baixo limite de detecção. O imunossensor tem grande potencial para ser utilizado como ferramenta de prevenção e redução de surtos causados por *Salmonella* e pode funcionar como base para o desenvolvimento de biossensores para detecção de outras espécies de bactérias.

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