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Full Length Research Paper

Biological evaluation of 32 different essential oils against *Acidovorax citrulli*, with a focus on *Cinnamomum verum* essential oil

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Bacterial fruit blotch (BFB) of watermelon caused by *Acidovorax citrulli* (ACC) is one of the most severe diseases of watermelon worldwide. Antibacterial activity of 32 essential oils (EOs) was evaluated against ACC using disk-diffusion assays. The oil from cinnamon exhibited the greatest antibacterial activity. Using gas chromatography-mass spectrometry (GC-MS), the major components of cinnamon oil were analyzed. Among the various components of cinnamon oil, benzaldehyde and cinnamaldehyde exhibited the effective antibacterial activities against ACC. The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of benzaldehyde and cinnamaldehyde were measured using broth dilution assays. The MICs against ACC of benzaldehyde and cinnamaldehyde were 0.1 and 0.01% (v/v), respectively. The MBCs of benzaldehyde and cinnamaldehyde against ACC were 0.2 and 0.02% (v/v), respectively. Also, 0.2% (v/v) levels of cinnamon oil, benzaldehyde and cinnamaldehyde completely killed ACC cells artificially contaminating watermelon seeds. This study suggests that cinnamon oil and its bioactive components, benzaldehyde and cinnamaldehyde, have potential for application as natural agents for the prevention and treatment of BFB.

Key words: *Acidovorax citrulli*, bacterial fruit blotch, cinnamon oil, essential oil.

INTRODUCTION

One of the most severe diseases of watermelon is bacterial fruit blotch (BFB), which is caused by *Acidovorax citrulli* (ACC). This disease is one of the major factors limiting yields worldwide (Burdman and Walcott, 2012). The disease was devastating and accounted for 100% loss of marketable fruit (Latin and Hopkins, 1995). Both watermelon seedlings and fruit are highly susceptible to BFB. A lag period occurs between infection and symptom development, and plants may thus remain asymptomatic for several days or more after infection (Burdman and Walcott, 2012).

A. citrulli can be introduced into watermelon fields in

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various ways, including contaminated seeds (Hopkins and Thompson, 2002) and infected transplants (Dutta et al., 2012), or via natural spread from alternate hosts such as wild cucurbits (Isakeit et al., 1998). *A. citrulli* may over-winter on infected wild cucurbits, volunteer plants or diseased plant debris. In the greenhouse, physical structures, equipment, and greenhouse supplies may be contaminated with the pathogen. The dense plant populations and high relative humidity, which is characteristic of greenhouse production facilities favor the spread of ACC. Overhead irrigation facilitates the spread of the pathogen among plants and can rapidly cause a large section of a greenhouse to become infected with ACC. Machinery, field workers, and wind-driven rain can spread ACC in the field. Hot and wet conditions in the field or greenhouse are critical environmental factors facilitating the spread of ACC and disease development. Grafting significantly increases the risk of ACC transmission (Burdman and Walcott, 2012).

The best form of control is to prevent the introduction of ACC into the field (Latin, 1996). Intensive efforts have been made by the seed and transplant industries to produce seeds and transplants that are free of ACC; such efforts have reduced the incidence of BFB. Despite these efforts, however, BFB outbreaks continue to occur every year, and BFB remains a significant problem worldwide (Hopkins, 1991; Burdman and Walcott, 2012). Currently, plant-derived essential oils (EOs) are highlighted as new generation antibacterial agents instead of antibiotics, which cause the appearance of antibiotics-resistance (Fabio et al., 2007; Samie et al., 2012; Seow et al., 2014; Hamedo, 2015). EOs are naturally occurring terpenic or aromatic mixtures, whose insecticidal and microbicidal actions against some plant pathogens have been reviewed (Isman, 2000).

The aim of the present study was to screen plant essential oils showing antibacterial activity against ACC and evaluate antibacterial activity of essential oils selected as active against ACC. *In vitro* antibacterial tests showed that cinnamon oil was the most effective against ACC. The major active constituents of cinnamon oil were determined via GC-MS, and the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the two most effective constituents were calculated. In addition, the *in vivo* antibacterial activities of these materials against ACC were investigated.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A. citrulli strain ACC02, which is highly pathogenic to watermelon plants, was used. The strain was cultured in Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L distilled water) with or without 1.5% (w/v) agar at 28°C. The strain was stored at -80°C for long-term storage.

Essential oils

Thirty-two EOs were purchased from HerbMall Co. Ltd., (Seoul, Korea). Supplementary Table S1 provides a list of the EOs used in the present study and the plant parts extracted.

Disk-diffusion assay

A bacterial suspension was prepared from an overnight-grown culture, and adjusted to an optical density at 600 nm (OD_{600}) of 0.5 (~1.0×10^8 CFU mL^{-1}) (or 0.5 McFarland turbidity units). A sterile swab immersed in the bacterial suspension was used to spread the entire surface of a LB agar plate. A total of 10 µL of each EO was applied to a sterile paper disc aseptically placed on the center of the inoculated plates. After 36 h of incubation at 28°C, the diameter of the zone of growth inhibition was measured in centimeters. Kanamycin served as a positive control. All experiments were carried out in triplicate. Average values of inhibition diameters were calculated to classify the EOs as follows: the strains were termed not sensitive (0) for a diameter smaller than 0.8 cm, moderately sensitive (+) for a 0.8–2.5 cm diameter, sensitive (++) for a 2.5–5 cm, and very sensitive (+++) for a diameter greater than 5 cm.

Gas chromatography-mass spectrometry (GC-MS) analysis

The active chemical constituents of the cinnamon oil were determined using gas chromatography-mass spectrometry (GC-MS), a GC puls-2010 coupled with GC-MS-QP2010 (Shimadzu, Japan), which was fitted with a HP-Innowax column (30 mm × 0.25 mm i.d. × 0.25 µm, J & W Scientific Co., USA). The temperature program started at 40°C for 1 min and increased to 250°C at 6°C min^{-1}, and then held for 4 min. Split injection (1:5 ratios) was performed with a 1-µL sample volume. The mass detector was fitted with an electron ionization source operated at 70 eV with a source temperature of 230°C. Helium was the carrier gas at a flow rate of 1 mL min^{-1}. Identification of EO compositions was based on the mass spectral information in a mass spectra library (McLafferty, 2000), and sample peaks were confirmed by comparison with the retention indices (RI) and mass spectra of authentic standards.

β-Phellandrene was prepared as described previously (Kang et al., 2013). Benzaldehyde, hydrocinnamic aldehyde and cinnamaldehyde were synthesized from the corresponding alcohol by PCC oxidation (Corey and Suggs, 1975). Hydrocinnamyl acetate and cinnamyl acetate were obtained by acetylation of the corresponding alcohol. Hydrocinnamyl alcohol was synthesized by hydrogenation of cinnamyl alcohol with Pd on the carbon.

Determination of MIC and MBC

MIC of the test compounds was determined using the broth dilution method in LB broth as described by Sfeir et al. (2013). Briefly, each compound was first diluted to 40% (v/v) in dimethyl sulfoxide (DMSO). Serial dilutions were carried out in sterile distilled water at concentrations of 0.01-0.5% (v/v). One milliliter of bacterial suspension (10^6 CFU mL^{-1}) and 0.1 mL of each compound showing antibacterial activity were added to 2.9 mL of LB broth. Controls without test compounds were prepared. After 24 h of incubation at 28°C under agitation in culture tubes, the MIC was determined as the lowest concentration that visibly inhibited bacterial growth.

To determine the MBC, 10 µL of bacterial inoculums were removed from tubes that had not presented visible turbidity and spread onto LB agar. These plates were incubated at 28°C for 48 h. The MBC was considered as the lower concentration that showed no bacterial growth on LB agar plates. Each MIC and MBC value was obtained from three independent experiments.

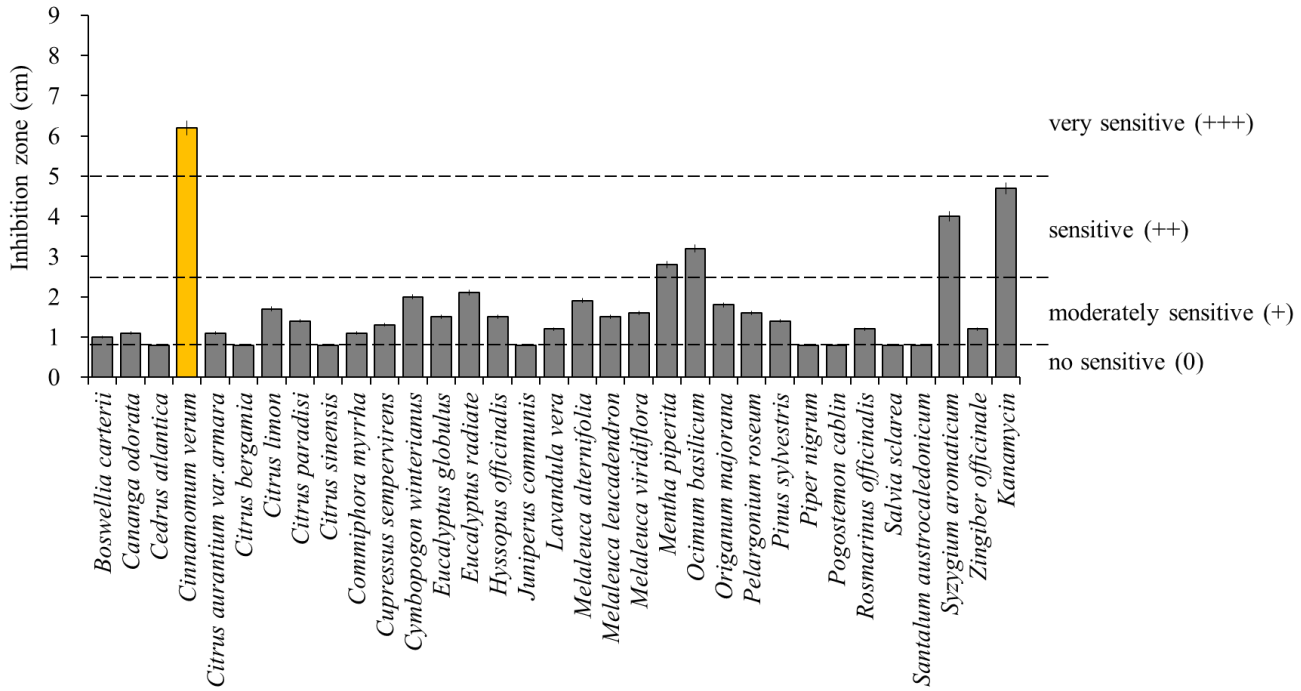

Figure 1. Inhibition zone diameters of the various essential oils against *A. citrulli* (means ± SD). Kanamycin (50 µg mL^{-1}) was used as the positive control. The experiments were carried out in triplicate. Average inhibition diameters were calculated to classify the EOs as follows: the strain was termed not sensitive (0) for a diameter smaller than 0.8 cm, moderately sensitive (+) for a 0.8–2.5 cm diameter, sensitive (++) for a 2.5–5 cm, and very sensitive (+++) for a diameter greater than 5 cm.

Antibacterial activity in watermelon seeds

Two chemical components (benzaldehyde and cinnamaldehyde) of cinnamon oil exhibited potent *in vitro* inhibition of ACC02 growth and were evaluated in terms of inhibition of ACC growing on artificially inoculated watermelon seeds. As an inoculum, a bacterial suspension was prepared from an overnight-grown culture, and adjusted to an optical density at 600 nm (OD_{600}) of 0.5 (1.0×10^8 CFU mL^{-1}) (or 0.5 McFarland turbidity units). Watermelon seeds (vr. Speed; Nongwoo Bio, Co. Ltd., Suwon, Korea) were soaked in the bacterial suspension for 30 min and dried.

Oil suspensions were prepared according to a previously described procedure with some modifications (Roh et al., 2011); 10 µL of cinnamon oil, benzaldehyde or cinnamaldehyde was dissolved in 1 mL ethanol followed by mixing with 9 mL distilled water to yield 0.1% (v/v) oil solutions, and Triton X-100 (0.009%, v/v) was added to each diluted solution. A mixture of ethanol (1 mL), Triton X-100 (0.009%, v/v), and distilled water (9 mL) served as a negative control. The seeds artificially contaminated with ACC were soaked in suspensions of cinnamon oil, benzaldehyde or cinnamaldehyde for 30 min, and dried. Three seeds per treatment were used in each experiment and bacterial colonies were calculated using a serial dilution plat method. The experiment was performed in triplicate.

RESULTS

Screening for antibacterial activity

Antibacterial activities of plant EOs against the ACC strain (ACC02) are presented in Figure 1. Results obtained from disk-diffusion assays showed that cinnamon oil was the most active against ACC02, with inhibition zones greater than 5.0 cm (+++). ACC02 was sensitive (++) to *Mentha piperita*, *Ocimum basilicum* and *Syzygium aromaticum* oils. Most EOs tested showed moderate inhibitory activities (+) against the tested strain. Eight EOs (those of *Cedrus atlantica*, *Citrus bergamia*, *Citrus sinensis*, *Juniperus communis*, *Piper nigrum, Pogostemon cablin, Salvia sclarea,* and *Santalum austrocaledonicum*) exhibited no significant activities (0) against the test strain. The inhibition zone of cinnamon oil was greater than that of the positive control, kanamycin.

Essential oil composition

The results of the chemical analysis are presented in Supplementary Table S2. The compounds are listed according to their elution order, which was in agreement with their RI on HP-Innowax columns (van Den Dool and Kratz, 1963). Of the 27 components of cinnamon oil, 24 were identified: α-pinene, camphene, β-pinene, 3-carene, α-phellandrene, α-terpinene, limonene, β-phellandrene, *p*-cymene, α-terpinolene, benzaldehyde, linalool, β-caryophyllene, humulene, α-terpineol, hydrocinnamic aldehyde, hydrocinnamyl acetate, caryophyllene oxide,

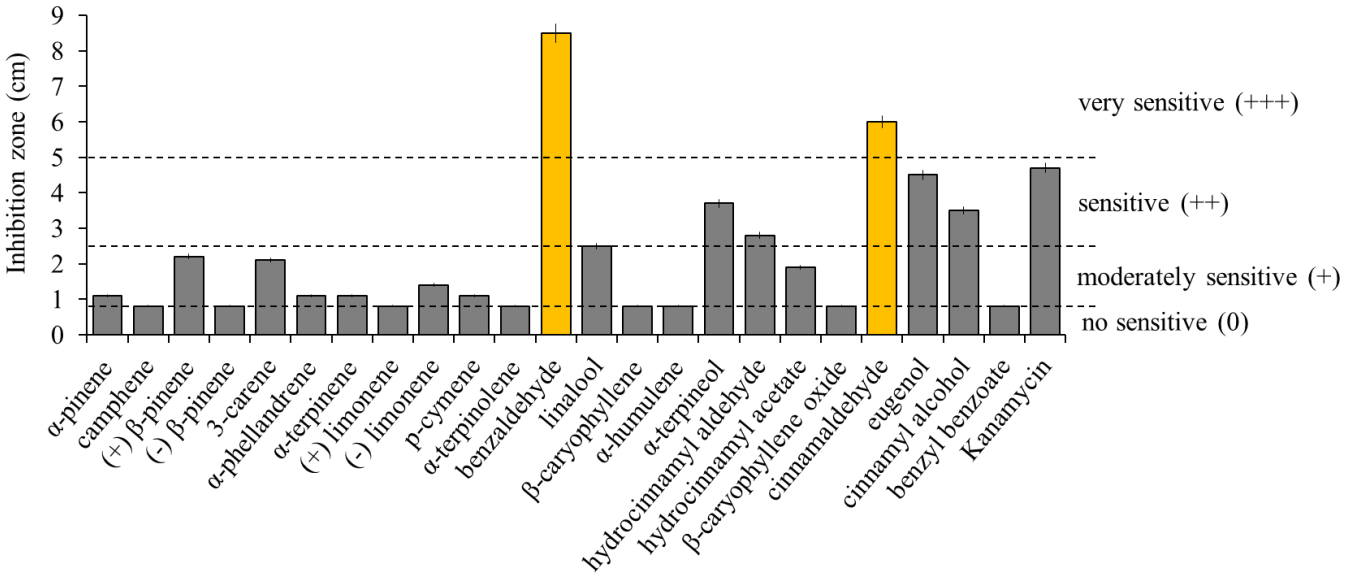

Figure 2. Inhibition zone diameters obtained for cinnamon oil components against *A. citrulli* (means ± SD). Kanamycin (50 µg mL^{-1}) was used as a positive control. The experiments were carried out in triplicate. Average inhibition diameters were calculated to classify the EOs as follows: the strain was termed not sensitive (0) for a diameter smaller than 0.8 cm, moderately sensitive (+) for a 0.8–2.5 cm diameter, sensitive (++) for a 2.5–5 cm, and very sensitive (+++) for a diameter greater than 5 cm.

cinnamaldehyde, cinnamyl acetate, eugenol, cinnamyl alcohol, methoxycinnamaldehyde and benzyl benzoate (Supplementary Table S2). Three peaks showed no match with the MS library. Cinnamaldehyde (44.35%) was the main compound in cinnamon oil, followed by β-phellandrene (9.55%) and cinnamyl acetate (8.5%) (Supplementary Table S2).

Antibacterial activities of cinnamon oil components

The antibacterial activities of the chemical constituents of cinnamon oil against ACC02 are presented in Figure 2. The results of the disk-diffusion assay showed that benzaldehyde and cinnamaldehyde were the most active against the tested bacterial strain, with inhibition zone diameters greater than 5.0 cm (+++). ACC02 was sensitive (++) to α-terpineol, hydrocinnamic aldehyde, eugenol and cinnamyl alcohol. The test strain was moderately sensitive (+) to α-pinene, (+) β-pinene, 3-carene, α-phellandrene, α-terpinene, (-) limonene, *p*-cymene, linalool and hydrocinnamyl acetate. No significant activity (0) was exhibited by eight compounds: camphene, (-) β-pinene, (+) limonene, α-terpinolene, β-caryophyllene, α-humulene, β-caryophyllene oxide and benzyl benzoate (Figure 2). The inhibition zones of benzaldehyde and cinnamaldehyde were greater than that of the positive control, kanamycin.

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values

Disk-diffusion assays for benzaldehyde and cinnamaldehyde were used to determine the most effective compound, and the MIC values were determined by means of broth dilution assays. The MICs of benzaldehyde and cinnamaldehyde were 0.1 and 0.01% (v/v) against ACC02, respectively (Figure 3). The MBCs of benzaldehyde and cinnamaldehyde against ACC02 were 0.2 and 0.02% (v/v), respectively (Figure 3).

Antibacterial activities on watermelon seeds

Cinnamon oil, benzaldehyde and cinnamaldehyde, exhibiting strong *in vitro* antibacterial activities against ACC02, were evaluated in terms of bacterial control on watermelon seeds. Each compound at 0.2% (v/v) completely killed ACC cells. At 0.05% (v/v), each compound inhibited bacterial growth by more than 70%. At 0.1% (v/v), cinnamaldehyde inhibited bacterial growth by more than 96% as compared to the control, whereas cinnamon oil and benzaldehyde inhibited bacterial growth by 75% (Figure 4).

DISCUSSION

Chemical control remains the major management in the control of plant diseases. One of the most important problems against the effective use of chemical control agents is the development and spread of resistant pathogens. The application of higher concentrations of various chemicals may increase the risk of high level toxic residues in the plant products. Despite the development of control agents, bacterial and fungal

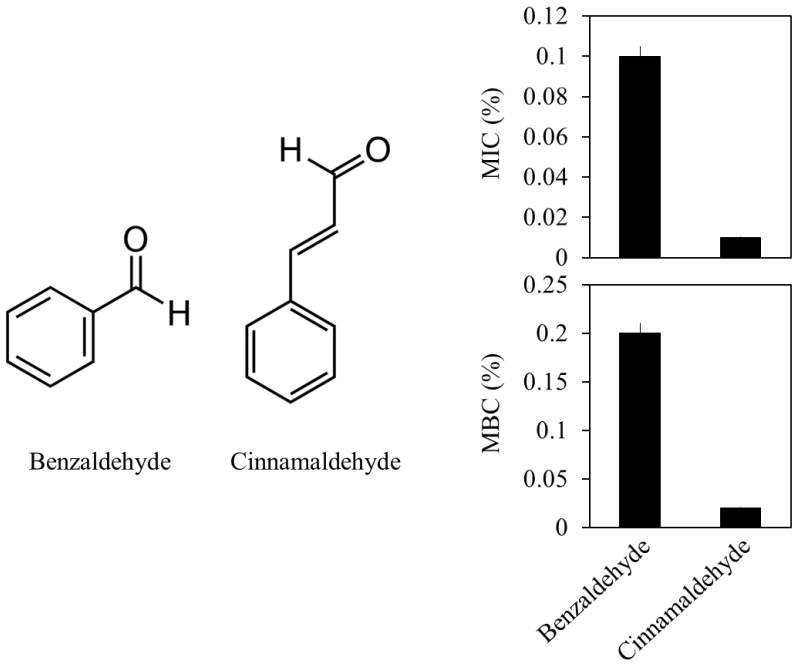

Figure 3. Chemical structure, minimum inhibitory concentrations and minimum bactericidal concentrations of benzaldehyde and cinnamaldehyde against *A. citrulli* (means ± SD).

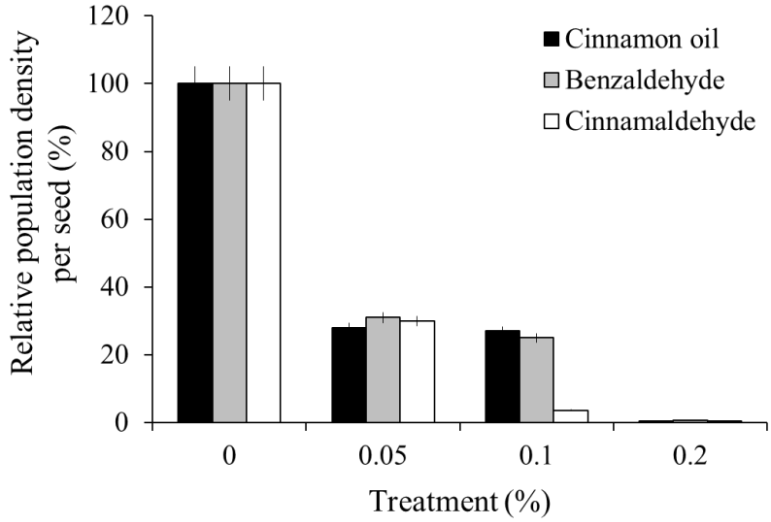

Figure 4. *In vivo* antibacterial activities of cinnamon oil, benzaldehyde and cinnamaldehyde against *A. citrulli* (means ± SD). The experiments were carried out in triplicate.

diseases are still a major problem in crop production. Therefore, there is an urgent need to develop new control agents, with higher activity, greater sensitivity, and lower toxicity. Plant-derived EOs are ideal for use in control formulations of plant diseases because they are antiseptic and environmentally friendly. In efforts to develop alternatives to synthetic chemicals for control of plant pathogens, interest in EOs has increased. However, scientific investigations to evaluate the antimicrobial activity of EOs are needed.

The present work evaluated the antibacterial activities of 32 EOs specifically against ACC, responsible for BFB

outbreaks. As a results, the EO of *C. verum* exhibited the greatest antibacterial activity against ACC. In the literature, the EO of *C. verum* was active *in vitro* against the following bacteria: *Escherichia coli*, *Staphylococcus aureus* (Barnes et al., 2007), *Streptococus mutans* (Fani and Kohanteb, 2011), *S. pyogenes* (Sfeir et al., 2013), *Salmonella typhimurium*, *Bacillus subtilis*, *B. thermoacidurans*, *Pseudomonas aeruginosa* (WHO, 1999) and *Helicobacter pylori* (Dugoua et al., 2007). However, no previous publications have reported the antibacterial activity of cinnamon oil against BFB-causing bacteria.

The chemical composition of cinnamon oil showing the greatest antibacterial activity against ACC was analyzed. GC-MS analyses indicated that cinnamaldehyde (44.35%) was the principal component of cinnamon oil extracted from the bark of *C. verum* plant; other major components were β-phellandrene (9.55%) and cinnamyl acetate (8.5%). The chemical composition of the cinnamon oil used in this study was very similar to that used in previous reports (Wang et al., 2005; Jeong et al., 2014). EOs containing mainly aromatic phenols or aldehydes have been reported to exhibit considerable antimicrobial activity, whereas EOs containing terpene ethers, ketones, or oxides had weaker activities (Inouye et al., 2001; Fabio et al., 2007).

In the present study, benzaldehyde and cinnamaldehyde exhibited effective antibacterial activities against ACC. These results are in agreement with previous reports showing that benzaldehyde and cinnamaldehyde have antimicrobial properties against several species of common foodborne bacteria (Bowles and Juneja, 1998; Helander et al., 1998). Cinnamon EO damages the cellular membrane of *Pseudomonas aeruginosa*, which leads to the collapse of membrane potential and loss of membrane-selective permeability. In *Staphylococcus aureus*, cells treated with the oil showed a considerable decrease in the metabolic activity and replication capacity, leading to a viable but noncultivable state (Bouhdid et al., 2010). Cinnamaldehyde exposure causes morphological changes in foodborne pathogenic bacteria, including *S. aureus*, *S. anatum* and *B. cereus* (Shan et al., 2007). However, the mode of action of active compounds of EOs has not been verified in the present study.

The antibacterial activity of cinnamon oil was investigated by determining the MIC and MBC values. For benzaldehyde and cinnamaldehyde, the MICs against planktonic ACC02 were 0.1 and 0.01% (v/v) (Figure 3). These high activities facilitated determination of MBC values. The MIC values were indeed low, and cinnamaldehyde was more effective against planktonic cells. The MBCs of benzaldehyde and cinnamaldehyde were determined using concentrations twice those of the MIC values to verify the accuracy of the MIC testing and to determine appropriate concentrations for use. Lobo et al. (2013) found that the MIC of cinnamon oil against *Streptococcus mutans* was 0.8 mg mL^{-1}. To the best of the authors' knowledge, no previous study has calculated the MIC or MBC of cinnamaldehyde against ACC.

EOs extracted from cinnamon bark had highly effective antibacterial activities against ACC. Cinnamon oil at 0.2% (v/v) completely killed ACC cells artificially contaminating watermelon seeds. Therefore, cinnamon oil can be used to control ACC on watermelon seeds. However, in the development of Eos as alternatives to synthetic bactericides, future studies must evaluate the phytotoxicities of EOs applied to plant seeds.

This study showed the *in vitro* and *in vivo* antibacterial activities of cinnamon oil and its active components benzaldehyde and cinnamaldehyde against ACC. In addition, our study gives a support for the application of cinnamon oil to eliminate ACC under specific conditions. However, for the development of cinnamon oil as an alternative of synthetic bactericides, further investigation should be carried out to obtain information regarding the practical effectiveness to protect plants without phytotoxicity.

Conclusions

This study showed that the EO of *C. verum* and its major components, benzaldehyde and cinnamaldehyde, possessed considerable *in vitro* antibacterial activities against bacterial fruit blotch of watermelon caused by *A. citrulli*. The MICs against ACC of benzaldehyde and cinnamaldehyde were 0.1 and 0.01% (v/v), respectively. The MBCs of benzaldehyde and cinnamaldehyde against ACC were 0.2 and 0.02% (v/v), respectively. Also, 0.2% (v/v) levels of cinnamon oil, benzaldehyde and cinnamaldehyde completely killed ACC cells artificially contaminating watermelon seeds. This study may be useful for application as natural agents for the prevention and treatment of BFB. Experiments that evaluate the effectiveness of EOs include: the ability to penetrate the seed coat; and the assessment to decontaminate cucurbit seeds without phytotoxicity.

Conflict of interests

The authors did not declare any conflict of interest.

Abbreviations

BFB, Bacterial fruit blotch; **ACC,** *Acidovorax citrulli*; **EO,** essential oil; **GC-MS,** gas chromatography-mass spectrometry; **MIC,** minimum inhibitory concentration; **MBC,** minimum bactericidal concentration.

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Supplementary Table S1. List of essential oils used in this study.

Plant	Plant species	Plant part
Bergamot	*Citrus bergamia*	Zest
Bitter orange	*Citrus aurantium* var.*amara*	Bud
Black pepper	*Piper nigrum*	Fruit
Blue gum	*Eucalyptus globulus*	Leaf
Cajeput tree	*Melaleuca leucadendron*	Leaf
Cedarwood	*Cedrus atlantica*	Wood
Cinnamon	*Cinnamomum verum*	Bark
Citronella	*Cymbopogon winterianus*	Grass and flower
Clary sage	*Salvia sclarea*	Leaf
Clove bud	*Syzygium aromaticum*	Bud
Cypress	*Cupressus sempervirens*	Branch
Eucalyptus	*Eucalyptus radiata*	Leaf
Frankincense	*Boswellia carteri*	Sap
Geranium	*Pelargonium roseum*	Aerial part
Ginger	*Zingiber officinale*	Rhizome
Grapefruit	*Citrus paradisi*	Zest
Hyssop	*Hyssopus officinalis*	Leaf
Juniper	*Juniperus communis*	Fruit
Lemon peel	*Citrus limon*	Zest
Myrrh	*Commiphora myrrha*	Flower and wood
Niaouli	*Melaleuca viridiflora*	Leaf
Patchouli	*Pogostemon cablin*	Leaf
Peppermint	*Mentha piperita*	Leaf
Rosemary	*Rosmarinus officinalis*	Leaf and flower
Sandalwood	*Santalum austrocaledonicum*	Wood
Scotch pine	*Pinus sylvestris*	Needle
Sweet basil	*Ocimum basilicum*	Flower and leaf
Sweet marjoram	*Origanum marjorana*	Flower and leaf
Sweet orange	*Citrus sinensis*	Zest
Tea-tree	*Melaleuca alternifolia*	Leaf
True lavender	*Lavandula vera*	Leading flower
Ylang-ylang	*Cananga odorata*	Flower

Supplementary Table S2. Chemical components of *C. verum* oil.

Component	Percentage (%)
Cinnamaldehyde	44.35
β-Phellandrene	9.55
Cinnamyl acetate	8.50
p-Cymene	6.31
α-Phellandrene	3.79
β-Caryophyllene	3.10
Limonene	3.06
Linalool	2.96
α-Terpinene	2.82
Eugenol	2.78
α-Terpineol	2.08
Unknown	1.27

Supplementary Table S2. Contd.

Benzyl benzoate	1.24
α-Pinene	1.23
Humulene	1.14
Camphene	1.00
Cinnamyl alcohol	0.89
Caryophyllene Oxide	0.73
Benzaldehyde	0.54
Hydrocinnamic aldehyde	0.40
Methoxycinnamaldehyde[2]	0.39
α-Terpinolene	0.37
β-Pinene	0.36
Unknown	0.32
Unknown	0.30
3-Carene	0.25
Hydrocinnamyl acetate	0.25

Quantification of each component was estimated by area normalization.

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Full Length Research Paper

Treatment of domestic wastewater by anaerobic denitrification: Influence of the type of support media on the production of extracellular polymer substances

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Eighteen Erlenmeyer flask containing six different support media [pozzolan, polyvinyl chloride1 (PVC1), polyvinyl chloride2 (PVC2), foam, polyethylene terephthalate (PET) and polystyrene (PS)] were subject to identical volumetric organic loadings and hydraulic retention time in treating synthetic protein ± carbohydrate waste. The objective was to examine the influence of support media on performance of anaerobic denitrification and retention and their resulting impact on system performance and failure. According to the results relative to every control support media, it was noticed that the best support media were the ones in PVC1 and PVC2, with successive reduction rates of 68.33 and 61.93% for chemical oxygen demand (COD), and 55 and 49% for nitrate. On the other hand, in two submerged anaerobic biofilter reactor packed with the support media of PVC1 and PVC2, the reactor with PVC1 media exhibited 89.93% COD and 78.75% nitrate removal efficiency attributable to its higher production of EPSp and EPSc.

Key words: Wastewater, anaerobic biofilm, extracellular polymeric substances (EPS), extraction, support media.

INTRODUCTION

Biologic denitrification of nitrates and nitrites present in wastewater is important and necessary. It is a process of nitrate and nitrite reduction in which nitrite serves as the terminal exogenous hydrogen acceptor when the oxygen tension in wastewater is sufficiently low. The normal end product of this nitrate and nitrite respiration is elementary nitrogen or nitrous oxide gas, which, being inert can be allowed to escape into the atmosphere (Narjari et al.,

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1984).

The literature containing numerous data has been reported in literature concerning the influence of different denitrification conditions on the rate of the process (Mazierski, 1984). Environmental conditions that must be optimized for denitrification are temperature, pH, and type of carbon substrate. In the present work, the system of biologic treatment was based on the oxidation of organic and nitrogen matter of synthetic wastewater. Micronutrients were added for good performance of microorganisms. Dissolved oxygen, pH, and temperature were monitored for the nitrification process.

Denitrifying bacteria are ubiquitous in nature (Gamble et al., 1977; Zumft, 1992), and biological denitrification treatment consists of the provision of suitable carbon and energy sources which may be organic or inorganic compounds. Treatment can take place in the aquifer, or the water may be pumped into above ground reactors.

Successful biological treatment of wastewater depends on the generation and maintenance of the appropriate sludge. The sludge is a complex dynamic biological structure composed of microflora (bacterial consortium) and micro fauna (mainly protozoa and metazoa). The bacteria naturally produce the extracellular polymeric substances (EPS) which form with bivalent cations a network where microorganisms are embedded (Wingender et al., 1999). Biofilms are dynamic environments, in which the microorganisms are optimally organized to make use of all available nutrients. In the biofilm, the EPS molecules provide the framework into which microbial cells are inserted, which is essential for the development of the architecture matrix (Sutherland, 2001).

Varesche et al. (1997) evaluated the anaerobic biomass attachment onto polyurethane foam matrices taken from the HAIB reactor treating a glucose-based substrate. The authors observed that polyurethane matrices offered excellent conditions for anaerobic growth and retention, due to the low level of microbial organization required by such a support material.

This paper presents some aspects of the influence of substrate on the process of biofilm formation onto polyurethane foam matrices. The results from the quantification of the biomass and extracellular polymers were used to investigate the role of substrate on the biomass adhesion onto polyurethane foam particles.

Substrate utilization in anaerobic filters has often been modelled based on fixed film fundamental. High media surface area seems to be desirable in AF applications for higher growth of biofilm. However, it has been reported that media surface area appears to have only a minor effect on the performance of upflow AFs (Young and Yang, 1989). The EPS are issued from bacteria metabolism and are considered as key components determining the physicochemical and biological properties of flocs and biofilms (Wingender et al., 1999; Flemming and Wingender, 2001).

This study has been initiated to examine the influence of support media on biomass growth and retention; either as suspended growth trapped within the interstitial void spaces or as attached biofilm adhered to the media surfaces in tow laboratory-scale anaerobic filters treating synthetic protein and some carbohydrate waste. The influence of the EPS on elimination of the chemical oxygen demand (COD) and the nitrate in treatment of domestic wastewater by anaerobic denitrification was evaluated quantitatively.

MATERIALS AND METHODS

Wastewater sampling

The origin of the poured residuary water in the sea comes from domestic wastewater or mixed with industrial wastewaters (95 and 5%). Samples of wastewater were collected and stored at 4°C (Figure 1).

Analytical design

Standard methods (AFNOR, 1986) were used for COD, nitrate and pH analyses of the samples (Table 1). The fixed biomass was quantified by the determination of EPS.

Support media used for biofilm adhesion

To study the effect of the nature of the medium on bacterial adhesion, six types of support were tested: pouzzolane irregularly shaped, foam (polyurethane) cube-shaped, two different types of poly vinyl chloride (PVC), polyethylene terephthalate (PET) and polystyrene (PS) in small rings, the characteristics are presented in Tables 2 and 3. The development of biofilm on these different media was followed in 250 ml Erlenmeyer flasks (with 3 replicates) containing the medium and the nutrient medium inoculated with denitrifying flora diluted 1/20. The anaerobic condition was ensured by keeping the media submerged, the flasks were kept tightly closed at room temperature. Denitrifying biomass was allowed to develop in the media for 5 days with the monitoring of the denitrification and the addition of KNO_3.

EPS extraction

A range of different chemical and physical approaches have been used to remove EPS from bacterial cell surfaces (Comte et al., 2006). Quantification of the major species of EPS (proteins and polysaccharides) was performed by colorimetric methods. Colorimetric method was based on the color developed by chemical reactions between the chemical functions of the molecule to be assayed and reagents. The color obtained is a function of the concentration of the species to be assayed. Measurement of the absorbance of the color was performed by UV visible spectroscopy.

Quantification of protein

In the case of wastewater, the choice of the most appropriate method for the quantification of proteins is not easy. However, the Lowry method was more frequently used in various scientific studies. The Lowry et al. (1951) method is based on two chemical reactions. First, the biuret reaction which involves the processing of

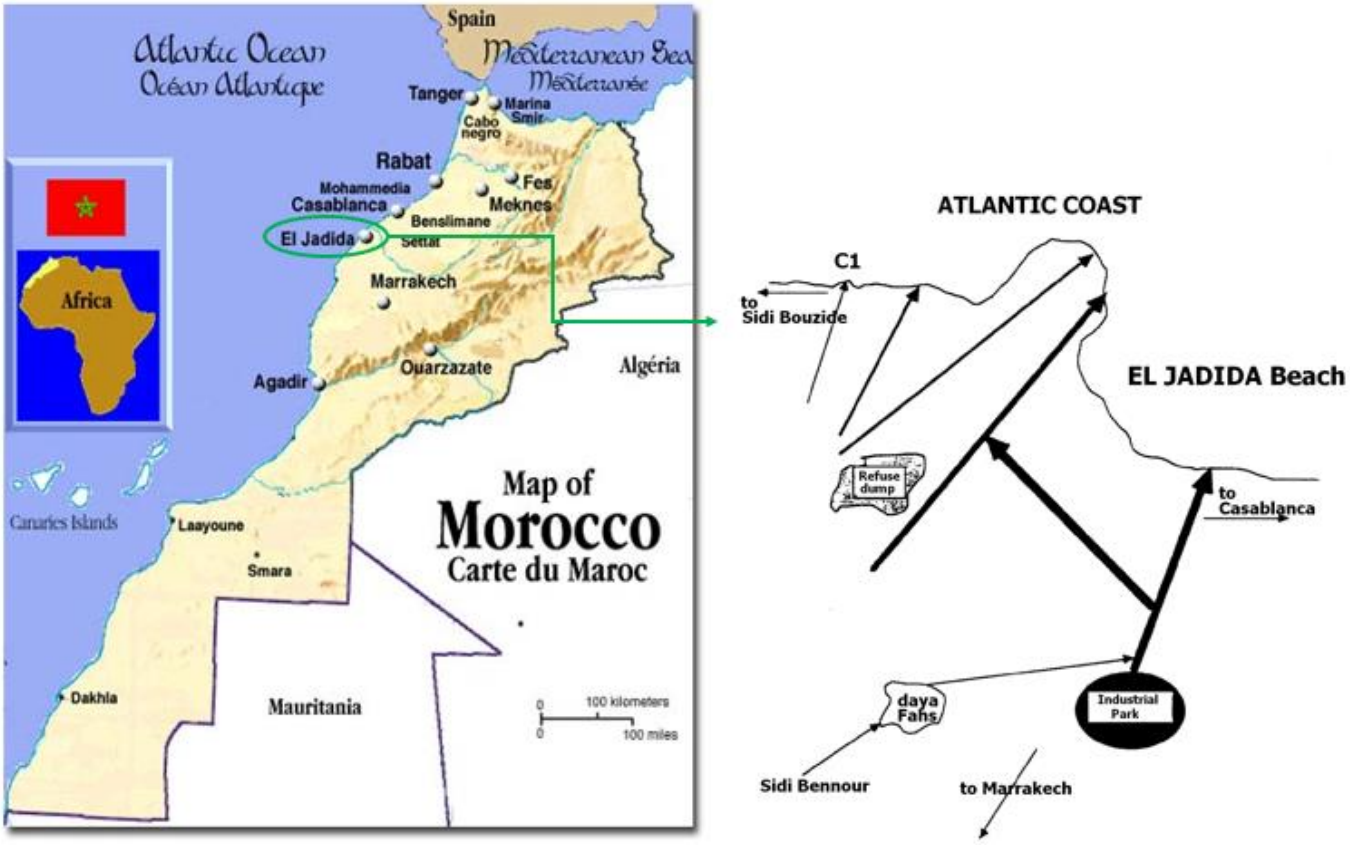

Figure 1. Location of sampling site in study zone: C1 (Lower town collector).

Table 1. Characterization average of pH, temperature, nitrate and COD in the effluent collected at the collector of sewage from the city of El Jadida.

Composition	Minimum	Maximum	Average	Standard deviation
pH	6.2	6.7	6.51	0.17
T°C	20	27	23.9	2.61
COD (mg/L)	545	624	575.2	28.12
Nitrate (NO^-_3) (mg/L)	1.90	4	2.9	0.68

Table 2. Characteristics of packing media in batch mode.

	Materials					
	Pouzzolane	**PVC1**	**PVC2**	**Foam**	**PET**	**PS**
Surface texture	Rigorous	Striated	Smooth	Rigorous	Smooth	Rigorous
Outside diameter (mm)	-	25	17	-	2	-
Height (mm)	12	12	25	18	30	20
Thickness (mm)	-	2	2	-	1	-
Specific surface (m^2/m^3)	115	99	187	292	957	324
Equivalent pore diameter (mm)	3.5	20	11	1	2	2

Table 3. Characteristics of packing media in continuous mode.

Materials		
	PVC1	**PVC2**
Surface texture	Striated	Smooth
Outside diameter (mm)	25	17
Height (mm)	45	25
Thickness (mm)	2	2
Specific surface (m^2/m^3)	99	187
Equivalent pore diameter (mm)	20	11

Figure 2. Experimental batch mode system.

peptide bonds with copper sulfate in alkaline solution. Second, the reduction of the complexes formed was carried out by the Folin-Ciocalteu. As a result, a blue solution was obtained. Measuring the absorbance of the solution formed was carried out by UV-visible spectroscopy at 750 nm.

Quantification of polysaccharides

The method used for the quantification of polysaccharides was that of Dubois al. (1956). In this method the polysaccharides were hydrolyzed through the heating by a strong acid (sulfuric acid). Then, saccharides reacted with the reagent specific to each method. The method of Dubois used phenol. This reagent produced the same intensity of color for all polysaccharides.

Reactor and batch mode system

Batch mode

Eighteen differential Erlenmeyer flask of 250 ml was used to evaluate the process of biofilm formation. Each erlenmeyer was filled with a media type (Figure 2). There, erlenmeyer was subjected to 260 ml of substrate with COD of 624 mg L^{-1} and 0.26 g KNO_3. The experiments were carried out in a batch mode. EPS was extracted from the media containing attached biomass using the cool aqueous extraction techniques (Sutherland and Wilkinson, 1971; Jia et al., 1991). The protein content of EPS (EPS_P) in the supernatant was measured according to the Lowry et al. method (1951) and the carbohydrate content (EPS_C) by the phenol/sulfuric-acid method. The sum of EPS_P and EPS_C represents the total EPS of the sludge.

Continuous mode

Tow 64.5 L columns packed with PVC1 and PVC2 ring was used as the anaerobic filters. Each reactor was 0.25 m in diameter and 1.05 m height, providing an empty bed of 64.5 L (Figure 3). The substrate was pumped into the bottom of the reactors through a variable speed pump “PERCOM N-M” Peristaltic and flowed upward

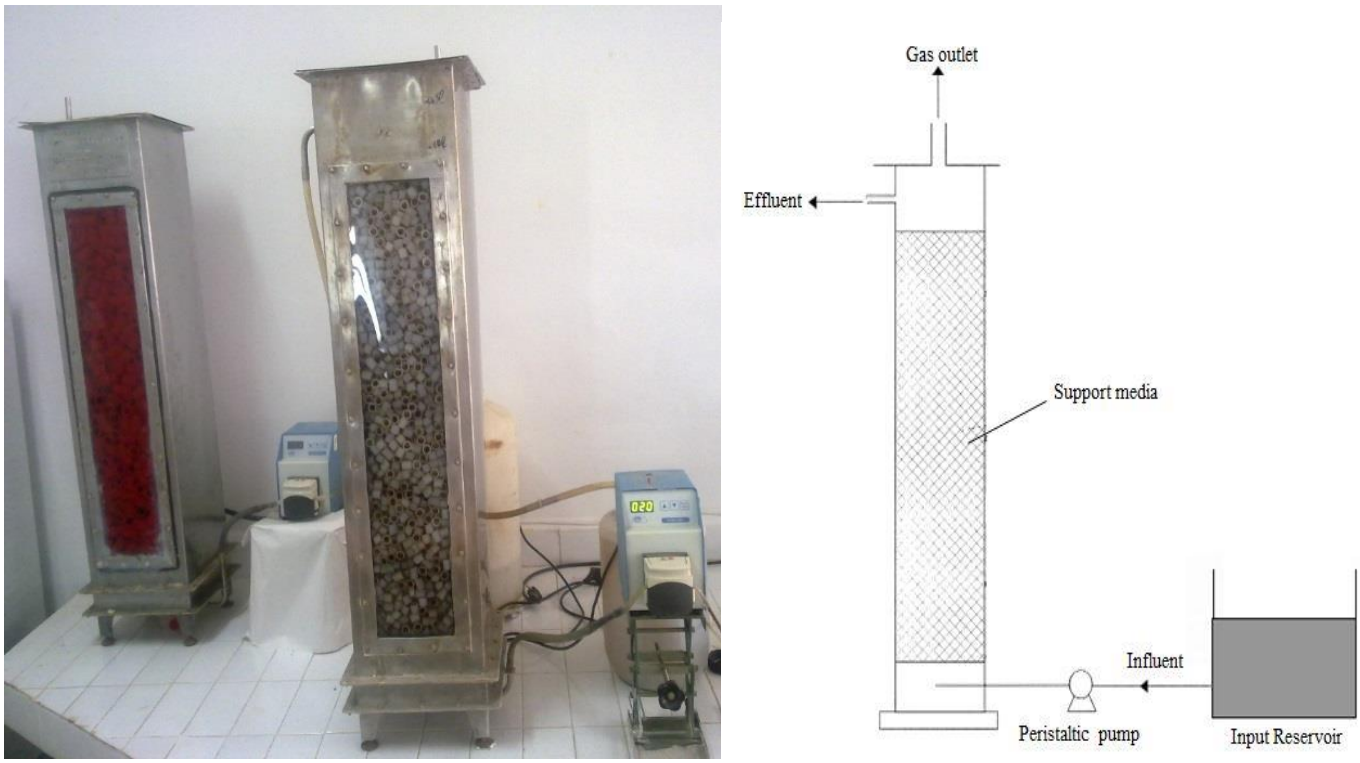

Figure 3. Experimental anaerobic bioreactor system.

through the porous medium. Sampling taps provided along the depth of the reactor allowed the extraction of samples for analysis at various stages of treatment. Both reactors were set at ambient temperature.

Denitrification studies and characterization of the isolates

This work has been the subject of a second study realized by our laboratory team. Denitrification experiments were performed in the medium, modified from Vossoughi et al. (1982), with the following composition (per liter of distilled water): $FeSO_4 \cdot 7H_2O$ 0.002 g, $CuSO_4 \cdot 5H_2O$ 0.02 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $MnSO_4 \cdot H_2O$ 0.12 g, $MgSO_4$ 0.8 g, $CaCl_2 \cdot 2H_2O$ 0.04 g, K_2HPO_4 3.2 g, yeast extract 0.3 g, KNO_3 4.7 g, sucrose 3 g, at pH 7.5. The isolates were grown in nutrient broth for 24 h and centrifuged at 10,000 rpm for 7 min. The cell pellet was washed twice with NaCl 0.9% and re-suspended in NaCl 0.9% with absorbance of 1.0 at 620 nm for isolates and 25 ml of this was inoculated in 250 ml Erlenmeyer flasks containing 250 ml of the medium. The flasks were incubated at 30°C under static conditions up to 6 days by sampling at an interval of every 24 h for estimating growth, nitrate, nitrite and organic matter. The identification of denitrifying strains was carried out on two isolates ADR1 and ADR2 sampled from an anaerobic bioreactor system. The isolates were subjected to 16S rRNA gene sequence analysis. A species level match is based on a similarity greater than or equal to 99% (Drancourt et al., 2000)

Isolate ADR1 was observed to be gram-positive bacilli, and BLAST results of partial 16S rRNA gene showed 99% identity with *Bacillus cereus*; isolate ADR2 gram-positive bacilli showed 99% similarity with *Bacillus tequilensis* with partial 16S rRNA gene sequence (Table 4). Phylogenetic positions of isolates are shown in Figure 4, where the isolates ADR1 and ADR2 clustered with *B. cereus* and *B. tequilensis*, respectively.

Table 4. Determination of denitrifying isolates by partial 16S rRNA gene sequence similarity.

Parameter	Isolate ADR1	Isolate ADR2
Similar species	*Bacillus cereus*	*Bacillus tequilensis*
Accession No.	KF484678	JX315319
% Similarity	99	99

RESULTS AND DISCUSSION

In batch mode

pH variation for each type of support media

The pH measured at the sampling point was 6.7. This value was consistent with the limit value of liquid discharges from the project dictated by Moroccan standards, which was between 6.5 to 8.5. The value of the temperature measured at the sampling point was consistent with the limit value of liquid discharges of the project dictated by Moroccan standards which sets a threshold temperature of 30°C for direct discharges and 35°C for indirect discharges.

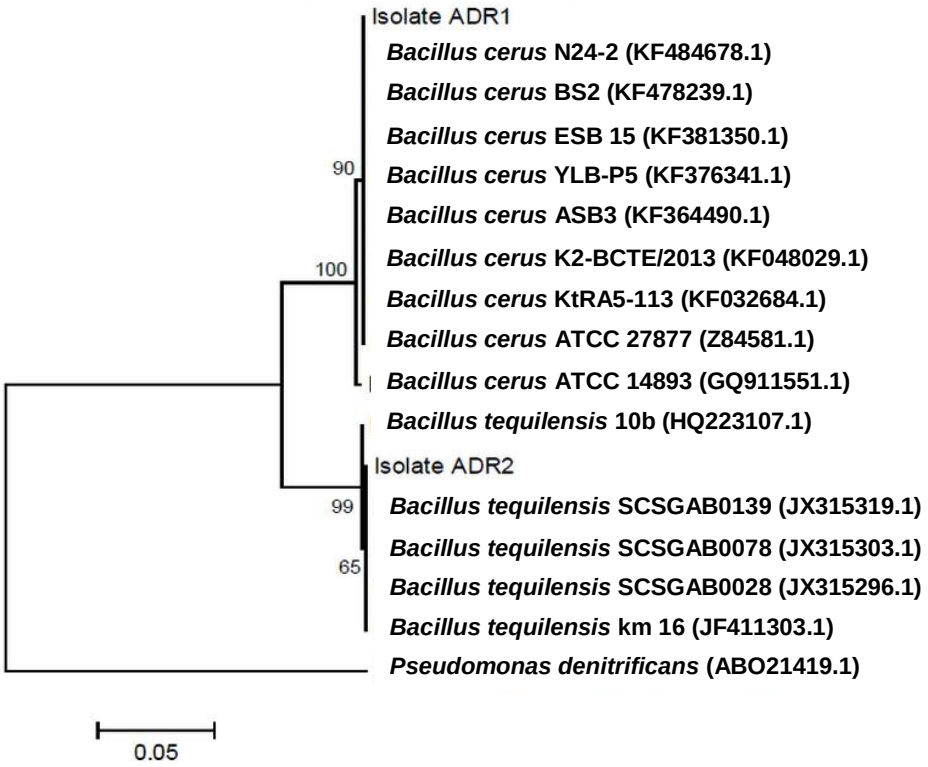

Figure 4. Phylogenetic tree constructed by neighbor-joining method showing position of the isolates with other related cultures. Bootstrap analysis of 10000 resampling by maximum-likelihood method was used to reconstruct tree. Parenthesis contains the accession number of the cultures. *Pseudomonas denitrificans* (AB021419) was used as an outgroup. Source: Moukhlissi et al. (2014).

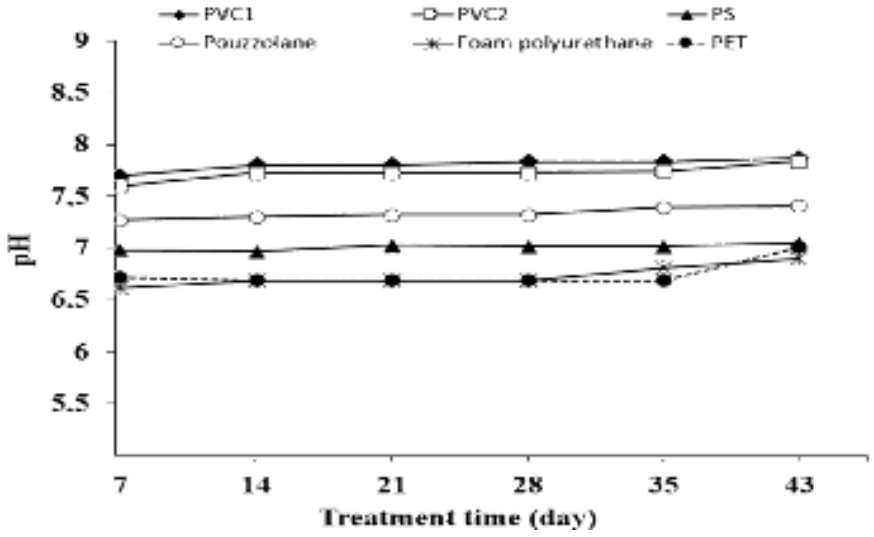

Figure 5. Evolution of pH in batch mode (initial pH is 6.1).

As shown in Figure 5, pH in influent was stabilized between 7.01 and 7.87, throughout the experiment. The optimum pH for denitrification is between 7.0 and 8.7 (Parkin et al., 1985; Šimeka et al., 2002). The pH in different Erlenmeyers with PVC1 then with the PVC2 increased from 7.6 to 7.87. For PS support media, the pH

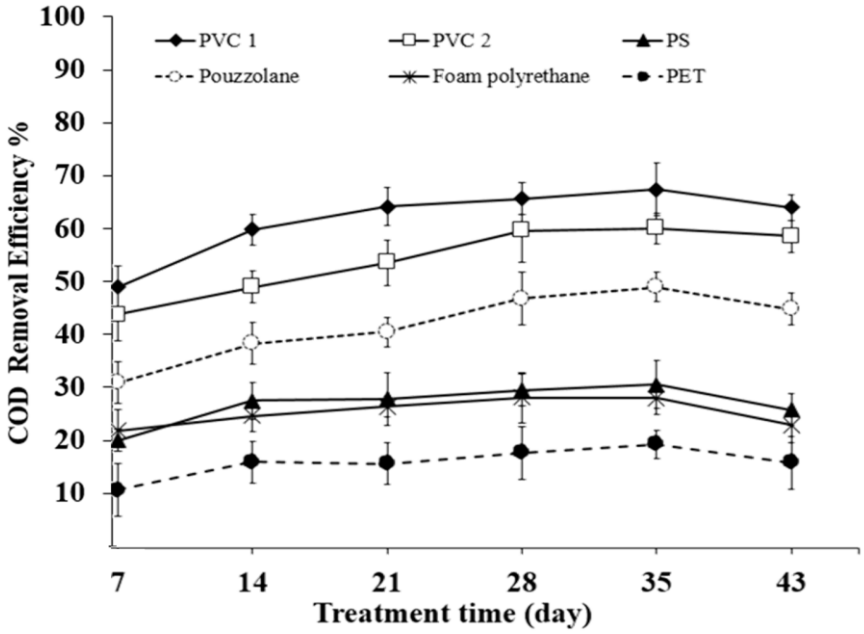

Figure 6. Steady-state COD removal efficiency.

was for the order of 7 and it remained constant. On the other hand, in different Erlenmeyers with Pozzolala, foam polyurethane and PET decreased on the whole from 6.98 to 6.69 to 28 days, and then it increased in 7.4 to 35 days and later it became stable. The degradation mechanism for the denitrification process could be deduced from the pH variation. The alkalinity and pH increased in heterotrophic and H_2-based autotrophic denitrification because nitrite reduction consumed protons (H^+). Proton consumption is illustrated in Equations (1 to 4) (Rittmann and McCarty, 2001).

Heterotrophic denitrification

$$NO_3^- + 0.263CH_3CH_2OH + 0.0445H^+ = 0.954NO_2^- + 0.0445C_5H_7O_2N + 0.655H_2O + 0.303\mathbf{CO}_2 \quad (1)$$

$$NO_2^- + 0.425CH_3CH_2OH + \mathbf{H}^+ = 0.455\ N_2 + 0.0912C_5H_7O_2N + 1.457H_2O + 0.393\mathbf{CO}_2 \quad (2)$$

Autotrophic denitrification

$$NO_3^- + 1.13H_2 + 0.01H^+ + 0.05\mathbf{CO}_2 = 0.99NO_2^- + 0.01C_5H_7O_2N + 1.1H_2O \quad (3)$$

$$NO_2^- + 0.122\mathbf{CO}_2 + \mathbf{H}^+ + 1.78H_2 = 0.488\ N_2 + 0.0244C_5H_7O_2N + 2.19H_2O \quad (4)$$

In both systems, nitrite reduction is the predominant source of alkalinity, consuming 1 H^+ equivalent per N equivalent of NO_2^- [highlighted by boldface in Equations (2) and (4)]. Another factor that affects pH is the net production of CO_2 in heterotrophic systems (highlighted by boldface in Equations 1 and 2 and net consumption of CO_2 in autotrophic systems (highlighted by boldface in Equations 3 and 4. CO_2 is a weak acid, and its addition partially suppresses the pH rise from proton consumption, as well as increases the concentration of total inorganic carbon species.

Evolution of COD removal

Effects of support media and times on purification efficiency was evaluated. As show in Figure 6, the COD removal efficiencies indicated that the support media PVC2 and PVC1 have significant purification efficiency at the loadings of 5 and 6 mg COD/L/day, with overall COD removal efficiency in excess of 60%. On the other hand, the support PET presents the lowest observed performance, with overall COD removal efficiency of 20%. This suggests that the anaerobic filters (AFs) can offer relatively high organic loading capacities compared with full-scale anaerobic contact plants which normally handle organic loadings of 1 to 3 g COD/L/day (Lawrence and McCarty, 1969). When the loading increased to 10 mg COD/L/day and subsequently to 15 and 25 mg COD/L/day, removal at the stages began to show relative superiority of support media PVC1 and PVC2 over foam polyurethane, pozzolana, PS and PET. The COD removal efficiencies was about 65% in both PVC1 and PVC2 and was 33, 36, 47 and 19% in PS, pozzolana,

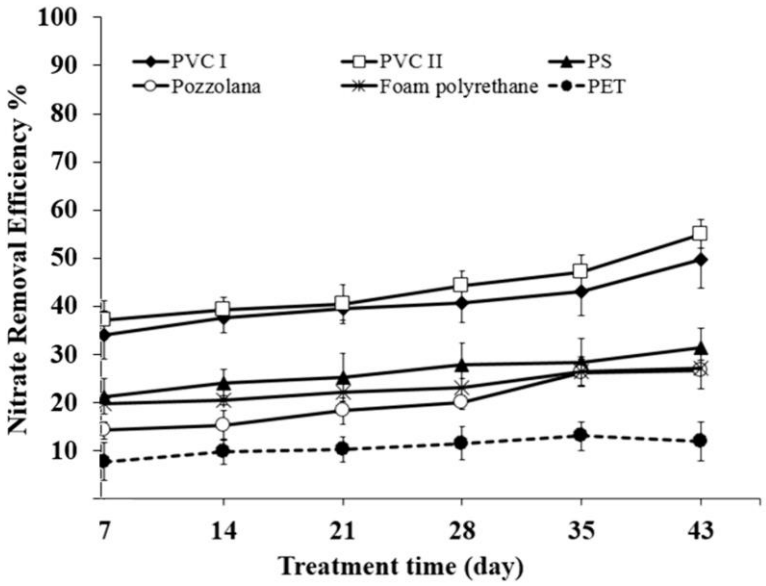

Figure 7. Steady-state Nitrate removal efficiency.

foam and PET, successively, at 10 mg COD/L/day. At higher loadings of 25 and 45 g COD and more, both PVC1and PVC2 showed similar COD removal of about 58.14 and 48.87% compared with the markedly reduced removal efficiencies of 5 and 57 in pozzolana.

The results indicated that this type of support media has a significant impact on the performance of purification efficiency. The higher removal efficiencies of PVC1 and PVC2 are likely attributed to higher growth of attached biofilm.

Evolution of nitrate removal

Biological denitrification is an efficient process for nitrogen removal from wastewater in which heterotrophic bacteria in the absence of oxygen (anaerobic conditions) convert nitrate-N and nitrite-N to nitrogen gas (Prosnansky et al., 2002; Van Rijn et al., 2006). As shown in Figure 7, the nitrate removal efficiencies indicated that all the erlenmeyers presented an important rate of elimination, but this elimination became stable between days 28 and 42. PVC1 and PVC2 presented a better performance in nitrate removing, with a maximum of 54.98%. On the other hand PET had low nitrate removal with 11.89% efficiency.

Evolution of the production of EPS

For years, carbohydrate was considered the main constituent of EPS in pure cultures (Sutherland, 2001; Sutherland and Kennedy, 1996). Recent studies of mixed cultures in wastewater treatment systems found that protein was also an important constituent in EPS, possibly due to the large quantities of exoenzymes entrapped in the EPS (Dignac et al., 1998). In this study, the protein content was greatest in the each type of media supports.

The production of EPSc and EPSp during all our experience showed similar evolutions (Figure 8). In the first phase, until the 21 days, a decrease of the ratio EPSp/EPSc was observed. This fact indicated that the EPS production can be related to adhesion of microorganisms onto the surface of each type of media support. Afterwards, it was verified that the ratio EPSp/EPSc decreased and kept constant until the end of last days of test. The findings were consistent with the results of the previous studies (Fenxia et al., 2011; Shim et al., 2001).

In continuous mode

pH variation for PVC1 and PVC2

As shown in Figure 9, pH in influent was stabilized between 8 and 8.1 in reactor with PVC2. The optimum pH for denitrification was between 7.0 and 8.7 (Parkin et al., 1985; Šimeka et al., 2002). In the reactor with PVC1, the pH increased from 8.3 to 8.7 towards 28 days.

Evolution of COD removal

COD conversion in all reactors was very high and stable during all the period of the experience. COD conversion from reactor with PVC1 was greater than 89.93 and

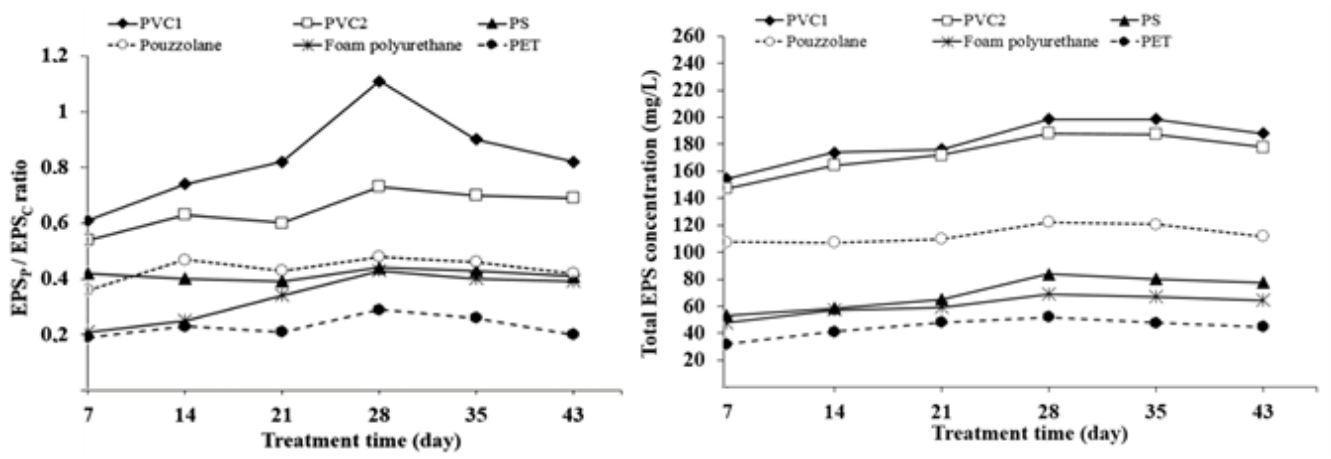

Figure 8. Ratio of EPSp/EPSc throughout the experiment.

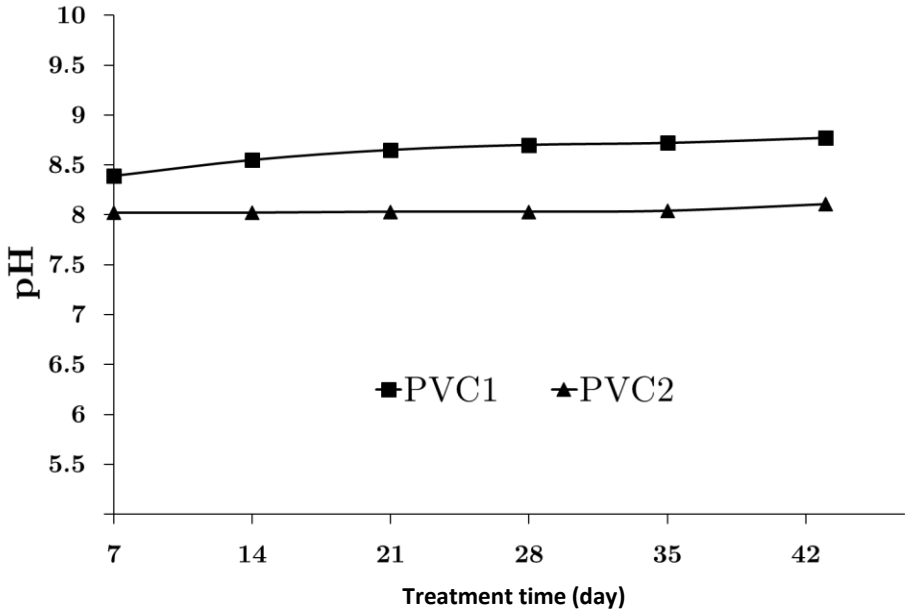

Figure 9. Evolution of pH in batch mode.

78.82% for reactor with PVC2 (Figure 10). The results indicated that the type of support media has a significant impact on the performance of purification efficiency. The higher removal efficiencies of PVC1 and PVC2 is likely attributed to higher growth of attached biofilm.

Evolution of nitrate removal

In this experience both reactors presented important performances with domination of the reactor with support media PVC1. As shown in Figure 7, The elimination of nitrate affected a maximum from 78.75% to 42 days for the reactor with PVC1. On the other hand, it was 66.81% for the reactor with PVC2 (Figure 11).

Evolution of the production of EPS in both reactors

The important factor determining the charge of the cell surface is the ratio of carbohydrates to protein in the EPS (Urbain et al., 1993). The production of EPSc and EPSp throughout the experiment showed similar evolutions (Figure 12). This production affected its optimum around day 35, with a ratio of 2.13 for the reaction with PVC2 and 2.22 for the reactor with PVC1. This resulted in an increase in the ratio of protein to carbohydrates, implying an important cell surface charge.

Denitrification pattern of the isolates

Based on 16S rRNA gene sequencing, isolates were

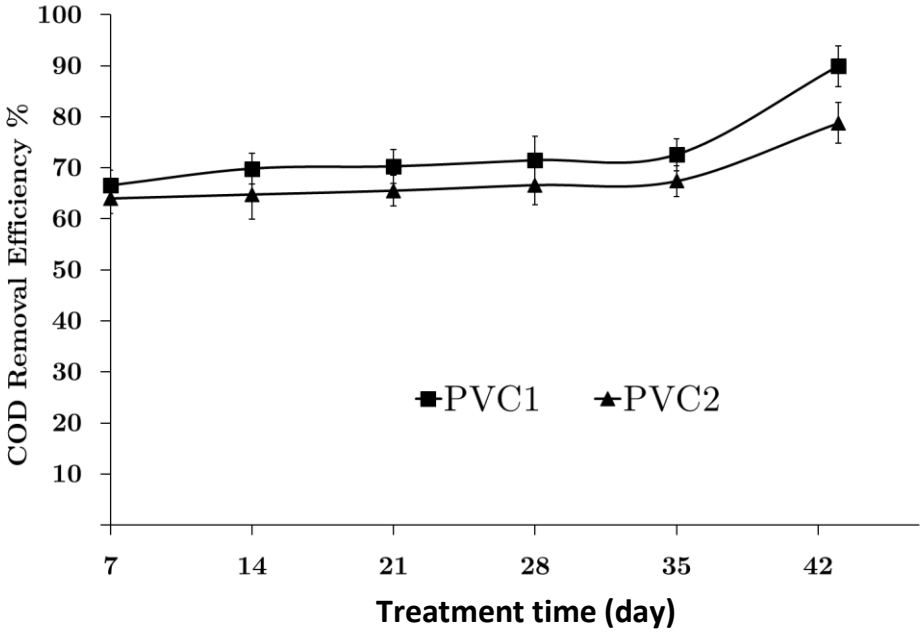

Figure 10. Evolution of COD removal efficiency.

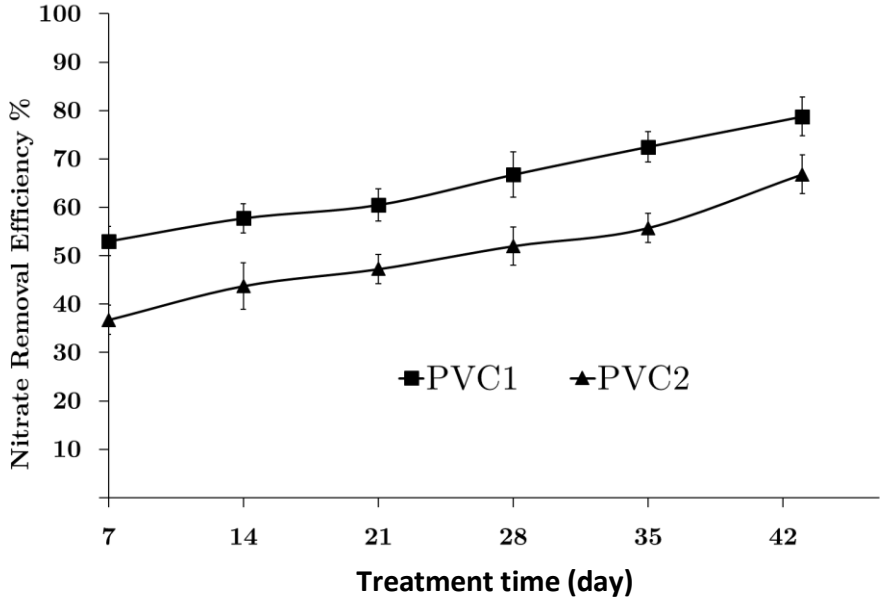

Figure 11. Evolution of nitrate removal efficiency.

affiliated with *Firmicutes*. The ability to denitrify has been identified in taxonomically diverse bacteria, including members of the *Aquificae*, *Deinococcus-Thermus*, *Firmicutes*, *Actinobacteria*, *Bacteroides* and *Proteobacteria* phyla (Zumft, 1997). Isolate ADR1 was related to *B. cereus*. *B. cereus* is a heterotrophic bacterium able to degrade organic matter under nitrate reducing conditions. Dou et al. (2010) reported that *B. cereus* could transform benzene to phenol and benzoate, and then used phenol and benzoate as carbon and energy source. Zhao et al. (2009) used the denitrifying *B. cereus* to remove nitrogen and organic matter from reclaimed wastewater used as landscape water. *B. cereus* is most likely involved in biogeochemical nutrient

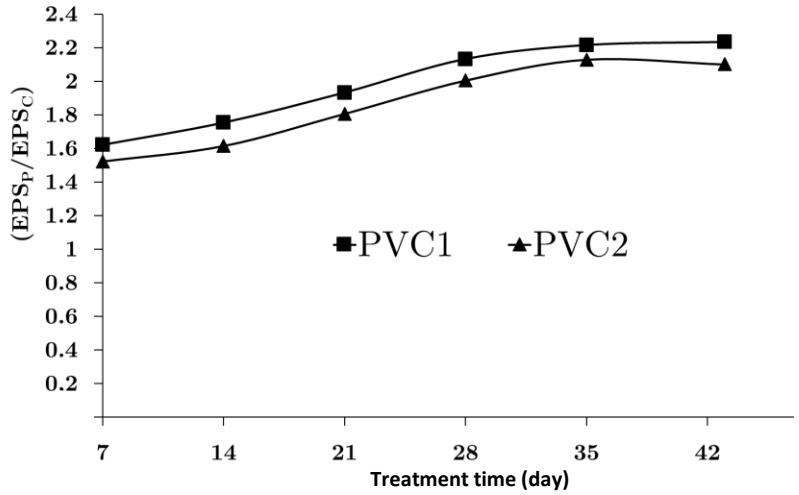

Figure 12. Variations of carbohydrate to protein ratio of EPS.

cycling, as it produces a wide range of extracellular enzymes and can grow on decaying organic matter (Borsodi et al., 2005). *B. tequilensis* could reduce nitrate to nitrogen, thus this species is a true denitrifier (Gatson et al., 2006). As reported by Das et al. (2014), *B. tequilensis* was chemoorganotrophic and could use hydrocarbons as sole carbon source.

Despite the fact that diverse denitrifiers have similar denitrification apparatus, each organism have its own activity. In this study, we compared the denitrification of two bacilli, ADR1 and ADR2, isolated from a denitrifying reactor and identified as *B. cereus* and *B. tequilensis*. The nitrate reduction rate was higher in *B. cereus*. However, two isolates have nitrite accumulation. Carlson and Ingraham (1983) revealed different patterns of denitrification between *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*. Betlach and Tiedje (1981) showed that transit nitrite accumulation in *Alcaligenes sp.* and *Pseudomonas fluorescens* was due to the differences in the reduction rates of nitrate and nitrite. The growth estimated by dry cell weight is more important in *B. tequilensis* than *B. cereus*. Otherwise, the increase of cell number leads to enhanced quantity of biomass and the sludge in the system of wastewater. Thus, *B. cereus* is more efficient because this strain reduces more amount of nitrate than *B. tequilensis* and produces less sludge.

The aforementioned results showed that isolates ADR1 and ADR2 isolated from denitrifying reactor were identified as *B. cereus* and *B. tequilensis*. The experimental results showed that *B. cereus* could reduce 29.46 mM of nitrate and degrade 4240 mg/L of organic matter within 6 days. However, *B. tequilensis* is less efficient and could reduce 13.82 mM of nitrate and 4500 mg/L of organic matter. In addition, *B. cereus* produced less biomass, avoiding clogging of wastewater treatment system. These results concerning *B. cereus* showed that the isolated bacterium could potentially remediate wastewater with high level of nitrate and organic matter. (Moukhlissi et al., 2014).

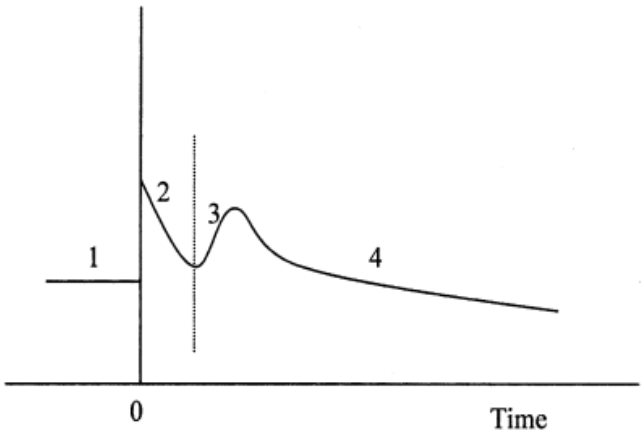

Figure 13. EPS biodegradability mechanisms: (1) pulse source of substrate from the added EPS; (2) easily biodegradable EPS was consumed; (3) produced soluble EPS plus minimally biodegradable EPS left; (4) newly produced EPS was further consumed and activity gradually stopped.

Conclusion

This study indicated that biofilm EPS was biodegradable by its own producers as well as by other microorganisms. Based on the aforementioned experimental evidence, we infer that the following events occurred during the EPS biodegradability study (Figure 13). The removal efficiency of COD and nitrate and production of EPS was closely

related with the type of support media. The PVC1 and PVC2 were favorable for the biofilm formation and therefore a better efficiency of wastewater treatment was obtained.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Iron bioavailability in tambaqui (*Colossoma macropomum*) desiccated gill and liver powder: Study in rats

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This study assessed iron bioavailability in rats from diets enriched with desiccated tambaqui gill and liver powders using the hemoglobin depletion-repletion method. Tambaqui (Colossoma macropomum) liver and gills were bought at Manaus. After processing the livers and gills were placed on trays and desiccated in a ventilated incubator at 60°C. The rats were given free access to chow and water. The moisture, protein, fat, and iron contents of the chow were determined three times. Iron bioavailability was measured by the hemoglobin depletion-repletion method. We used 24 anemic animals, which were randomly selected and distributed into three groups of eight animals each: 1) Control Group – anemic rats fed a casein-based diet (AIN 93); 2) Experimental Group – anemic rats fed AIN 93 and desiccated tambaqui liver; 2A) Experimental Group – anemic rats fed AIN 93 and desiccated tambaqui gill; and 3) Pair-feeding Group – anemic rats, distributed in random blocks according to hemoglobin concentration and weight, fed the average amount of AIN 93 consumed by the Control group. Gills are high in lipids. In addition to high lipid content, gill powder had considerable levels of protein and iron. The baseline hemoglobin of Groups 2 (liver) and 2A (gills) did not differ. After seven days, only Group 2 (liver) reached appropriate hemoglobin levels. In conclusion, iron in desiccated tambaqui liver powder is highly bioavailable. The iron in desiccated tambaqui gill powder is not as bioavailable as rats consuming this powder did not reach appropriate hemoglobin levels within the experimental period.

Key words: Bioavailability, iron, powder, liver, gills, tambaqui.

INTRODUCTION

Anemia is considered one of the great global public health problems, especially iron-deficiency anemia, responsible for 95% of all anemia cases (Torres et al., 1994). According to World Health Organization (OMS, 2001) estimates, approximately 50% of all children aged less than four years in developing countries are anemic, representing a severe human health problem as 55% of child deaths are related to malnutrition. Despite the commitment made by 170 countries, including Brazil, during the World Summit for Children held in 1992 in Rome, to prioritize the fight against iron-deficiency anemia, the problem persists. Fisberg et al. (2001) found

that 54% of children from 10 Brazilian capitals aged less than five years had iron deficiency. This situation led the National Sanitary Surveillance Agency to pass the National Policy for Brazilian Food and Nutrition, RDC n°. 344, on December 13, 2002. This policy aims to reduce iron-deficiency anemia by establishing the compulsory enrichment of wheat meal and corn flour with iron and folic acid (ANVISA, 2002). However, assessing iron bioavailability in ingredients is more important than fortifying foods with iron as high concentrations of iron do not necessarily translate to high utilization rates by the human body (Troari et al., 2005).

Approximately 217,000 tons of fish are caught annually in the Amazon (Val and Santos, 2009), and households in the region consume approximately 30.0 kg of fish per year, as opposed to 4.0 kg/year by Brazilians in general (IBGE, 2010). Therefore, fish is the main source of animal protein in the Amazon (Oetterer, 2006). Manaus is the main port of delivery for all this catch. The large amounts of byproducts obtained by processing can be used as raw material for feeds and add value to other products (Stori et al., 2002). Brazil discards approximately 50% of fish biomass (Pessatti, 2001). Using residues may solve the pollution problem caused by a substance that is difficult to discard and instead generate economic, social and environmental especially public health benefits. The present study chose tambaqui's (*Colossoma macropomum*) because it is the most cultivated species in the Amazon region (IBAMA, 2007), with an estimated production of 14,000 tons/year (Inoue and Boijink, 2011). Since large amounts of tambaqui liver and gills are discarded, the study hopes to find ways to use these outstanding iron sources, which may become very important in the fight against deficiencies, especially iron. Adequate iron intake depends not only on individual requirement but also on iron bioavailability in different foods. Although there is little information about the use of tambaqui liver and gills, we estimate that approximately three tons of these byproducts are discarded daily in Amazon Rivers. Although organs are degradable, large amounts pollute the environment and unbalance the ecosystem. Hence, the study aimed to process tambaqui gills and liver, to determine the nutritional constituents of their powders, and to measure their iron bioavailability in an experimental rat model.

MATERIALS AND METHODS

Tambaqui (*C. macropomum*) liver and gills were bought at Manaus' farmer's markets, placed in coolers, and transported to the Laboratory of Food and Nutrition – Laboratório de Alimentos e Nutrição/ Instituto Nacional de Pesquisas da Amazônia (LAN/INPA), Brazil. The organs were rinsed with tap water, boiled in a stainless steel pot for 10 min, dehydrated by placing even slices of approximately 1.0 cm of livers and gills on trays, and desiccated in a ventilated incubator at 60°C until the weight stabilized, indicating the moisture content. The samples were then ground by an electric grinder. The powder was stored in polyethylene packages until physical, chemical and microbiological analyses. In order to minimize metal contamination, especially iron, all glassware and utensils were rinsed with a 30% solution of nitric acid, rinsed with deionized water, and dried at least six times. Casein-based chows were prepared exactly as recommended by the American Institute of Nutrition – AIN-93G (Reeves et al., 1993). The animals had free access to food and water.

The moisture, protein, lipid (AOAC, 1995) and iron contents of the chow were determined three times by atomic absorption spectroscopy, as recommended by Institute Adolph Lutz (IAL, 2008), using the method provided by the Varian manual (VARIAN, 2000). The samples were digested in the microwave digester MARS (Xpress CEM Corporation, MD – 2591). The organic material was mineralized by concentrated nitric acid, cooled and diluted with deionized water. The iron contents of the diluted solutions were determined directly by atomic absorption spectroscopy (Spectra AA, model 220 FS, Varian, 2000), with specific lamps as instructed by the manufacturer. The analyses were controlled as instructed by the Varian Manual (Cornelis, 1992), using certified Peach leaves (NIST – SEM 1547) as reference.

The levels of *Salmonella*, total coliforms, fecal coliforms and *Escherichia coli* of the samples were determined by the International Commission on Microbiological Specifications for Foods (ICMSF) method, as required by RDC 12/01 (ANVISA, 2001) of the National Sanitary Surveillance Agency (ICMSF, 1983). This project was approved by the Animal Research Ethics Committee of the Federal University of Amazonas (UFAM) under protocol number 068/2012.

Iron bioavailability was determined by the hemoglobin depletion-repletion method. Wistar dams (*Rattus norvegicus* albinus, Rodentia, Mammalia) with six pups each (n=42) provided by the Animal Facility of the National Central Institute of Amazon Researches (INPA) were fed a casein-based chow without iron added to induce iron-deficiency anemia during the nursing period (21 days). After weaning, the pups were fed the same chow for another seven days. At the end of the depletion stage, blood was collected by sectioning the terminal portion of the tail to determine hemoglobin and select animals for the repletion stage. We used the cut-off points suggested by Margoles (1984): Anemia in rats is defined as Hb<7 g/dL and normal iron status as Hb>11 g/dL. For the 14-day iron repletion stage, anemic animals were randomly distributed into three groups with eight rats each: (1) Control group - anemic rats fed a casein-based diet (AIN 93); (2) experimental group - anemic rats fed AIN 93 and desiccated tambaqui liver powder as iron source; 2A) experimental group - anemic rats fed AIN 93 and desiccated tambaqui gill powder as iron source; and (3) Pair-feeding group - anemic rats fed the average amount of AIN 93 consumed by the control group, distributed into random blocks according to hemoglobin level and weight. During the iron depletion stage, the animals were housed in polypropylene boxes with

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#In memoriam.

Table 1. Chemical composition of the chows used during iron depletion and repletion stages, Manaus (AM), 2013.

Ingredients	AIN 1993 G (%)*	AIN 1993 G (%)**	Liver powder (%)**	Gill powder (%)**
Casein	20.00	20.00	18.40	18.40
Sucrose	10.00	10.00	10.00	10.00
Soybean oil	7.00	7.00	7.00	7.00
Microcrystalline fiber	5.00	5.00	5.00	5.00
Saline	-	3.50	-	-
Saline without iron	3.50	-	3.50	3.50
Vitamin mix	1.00	1.00	1.00	1.00
L-cysteine	0.30	0.30	0.30	0.30
Choline bitartrate	0.25	0.25	0.25	0.25
Liver flour	-	-	4.00	-
Gill flour	-	-	-	4.00
Subtotal	`47.05	47.05	49.45	49.45
Corn starch	52.95	52.95	50.55	50.55
Total	100.00	100.00	100.00	100.00

*Chow during the depletion stage; **chow during the repletion stage; AIN 1993 G=American Institute of Nutrition.

Table 2. Proximate composition of desiccated tambaqui (*Colossoma macropomum*) liver and gill powders in 100 g of dry base. Manaus (AM), 2013.

Parameter	Tambaqui liver powder	*Raw beef liver	*Raw chicken liver	Tambaqui gill powder
Moisture (g)	2.77**	71.3	77.8	0.49**
Ash (g)	3.53	1.5	1.2	6.65
Proteins (g)	39.82	20.7	17.6	31.33
Lipids (g)	17.81	5.4	3.5	56.63
Iron (mg)	86.74	5.6	9.5	25.78

*Source: Brazilian Food Composition table (TACO). Food Study and Research Core (*Núcleo de Estudos e Pesquisa em Alimentação*, NEPA), UNICAMP. v.2. 4. ed. Campinas, 2011. 161 p. ** Residual moisture.

stainless steel lids, and in the repletion stage, they were housed in individual stainless steel cages under controlled humidity and temperature (~23°C) and 12 h light/dark cycles. The rats had free access to food and water. The chows used in the experimental period were prepared as recommended by Reeves et al. (1993) at Table 1, with 35 mg of iron/kg of chow. At the beginning and end of each repletion week, blood was collected by caudal vena cava puncture. Hemoglobin was determined by Hemo-Control microcuvettes and directly by the portable hemoglobinometer HemoCue®.

The results were submitted to analysis of variance (ANOVA). Statistical analyses were conducted by the software INSTAT version 3.0 and included the Tukey-Kramer comparisons at a significance level of 5% (Gomes, 1987).

RESULTS AND DISCUSSION

Salmonella sp. and total and fecal coliforms were not found in any of the samples. These findings confirm good manufacturing practices and proper hygienic conditions during processing, which is in agreement with Resolution RDC no. 12 passed on January 02, 2001 (Brasil, 2001). Table 2 shows the proximate composition of desiccated tambaqui gill powder. The gills have high lipid content, 56.63 g in 100 g of edible parts. For comparison, raw beef liver contains 5.4 g of lipids in 100 g of edible parts (TACO, 2011). Gill powder also contained high protein and iron contents. On the other hand, desiccated tambaqui liver powder has higher iron and protein contents than other foods (Table 2). The nutritiousness of this organ with respect to iron, lipids, and proteins is undeniable. At the end of the depletion period, the mean hemoglobin of the animals fed a low-iron chow was significantly lower, demonstrating that the methods were appropriate for the study objectives. These results are similar to laboratory results that used the same methods (Silva et al., 1998). During the repletion stage, the baseline hemoglobin of groups 2 and 2A (liver and gills) did not differ ($p<0.05$). On day seven, only the group consuming desiccated tambaqui liver powder reached normal hemoglobin levels ($p<0.05$). On day fourteen, all rats had higher hemoglobin levels ($p<0.05$), indicating the bioavailability of iron in desiccated tambaqui liver powder

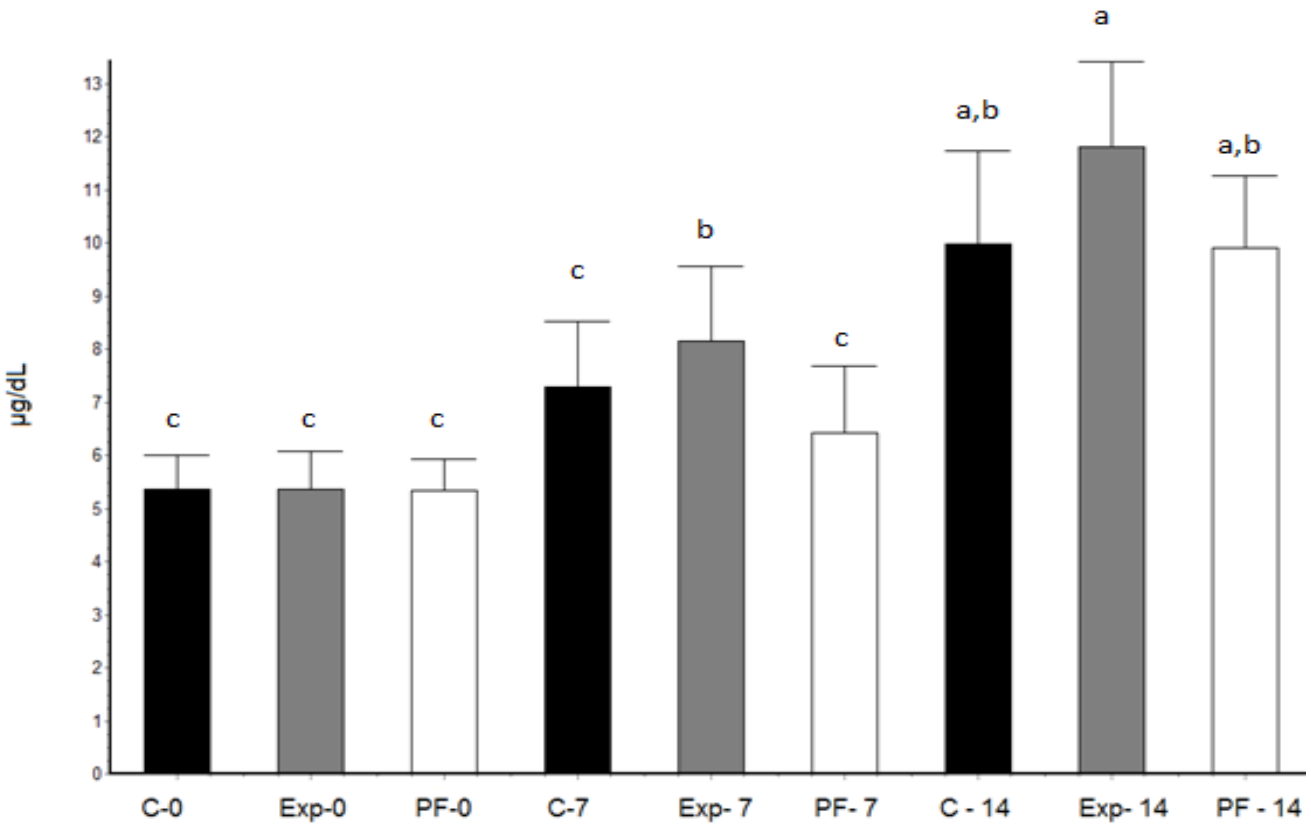

Figure 1. Hemoglobin levels (Hb) of animals in the control group (C), experimental group 2 (liver, Exp), and paired-feeding group (PF) at baseline (0), on day seven, and on day fourteen. Levels with the same letters are not significantly different according to the Tukey test ($p<0.05$).

(Figure 1). At baseline, the hemoglobin levels of the animals in the desiccated tambaqui gill powder group were not different from those of the other groups. However, on day seven, the hemoglobin levels of the groups differed significantly. On day fourteen, the hemoglobin levels of the experimental groups differed significantly from those of the control and pair-feeding groups, and the hemoglobin levels of all groups differed from those at baseline and on day seven (Figure 2). At baseline, all rats had similar weights. On days seven and fourteen, the rats in the group pair-feeding had gained significantly less weight than those in the other three groups ($p<0.05$) (Figure 3). The body weight of the rats that consumed desiccated tambaqui gill powder did not differ from that of the other groups at baseline and on day seven. However, on day fourteen, the control group differed from the other groups and from itself at baseline and on day seven (Figure 4).

Conclusion

In conclusion, iron in desiccated tambaqui gill powder is not as bioavailable, not helping anemic animals to recover normal hemoglobin levels. Thus, desiccated tambaqui gill powder as an iron source should be used with caution. On the other hand, iron in desiccated tambaqui liver powder is highly bioavailable, so other studies should assess the impact of adding this food to the diet of preschoolers and groups at risk of anemia, which would provide a new, alternative, and healthy source of dietary iron for Amazonians.

Conflict of Interests

The authors have not declared any conflict of interests.

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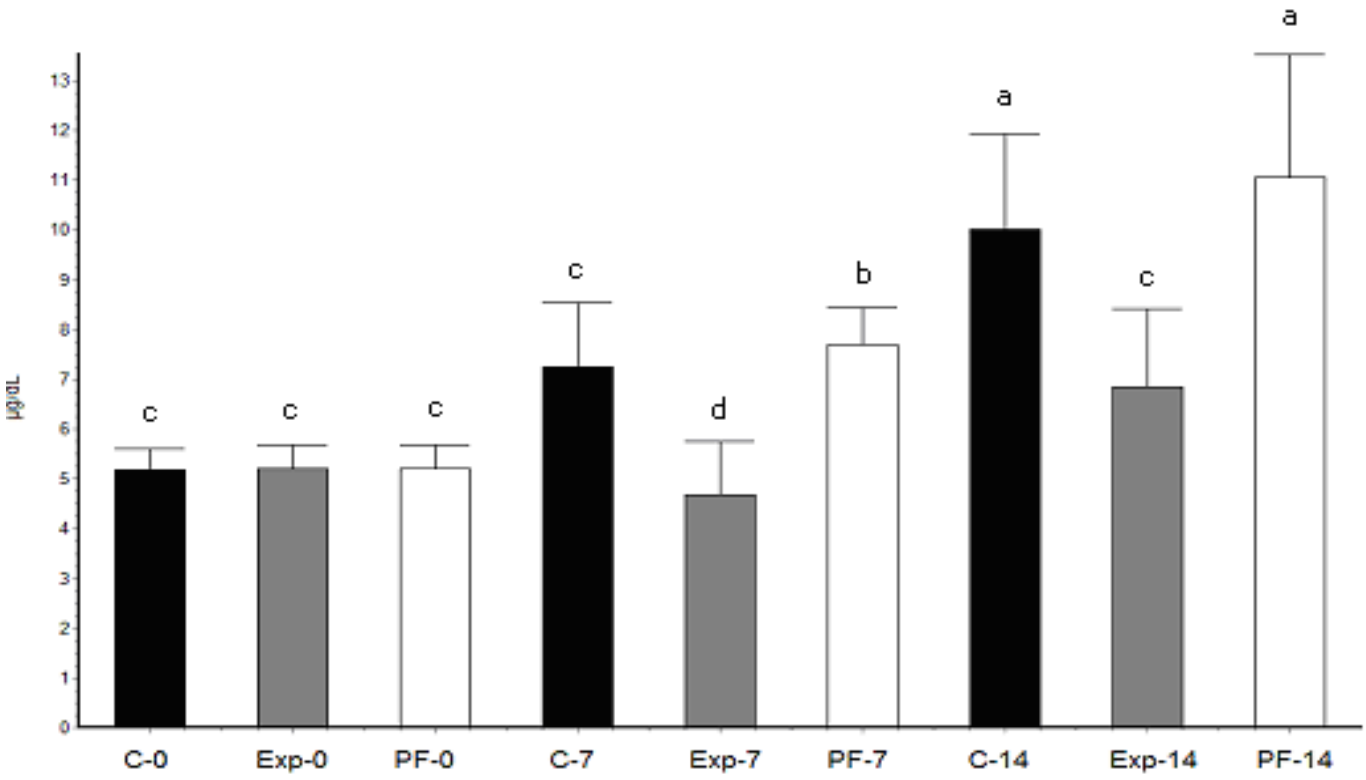

Figure 2. Hemoglobin levels (Hb) of animals in the control group (C), experimental group 2A (gills, Exp), and paired-feeding group (PF) at baseline (0), on day seven, and on day fourteen. Levels with the same letters are not significantly different according to the Tukey test ($p<0.05$).

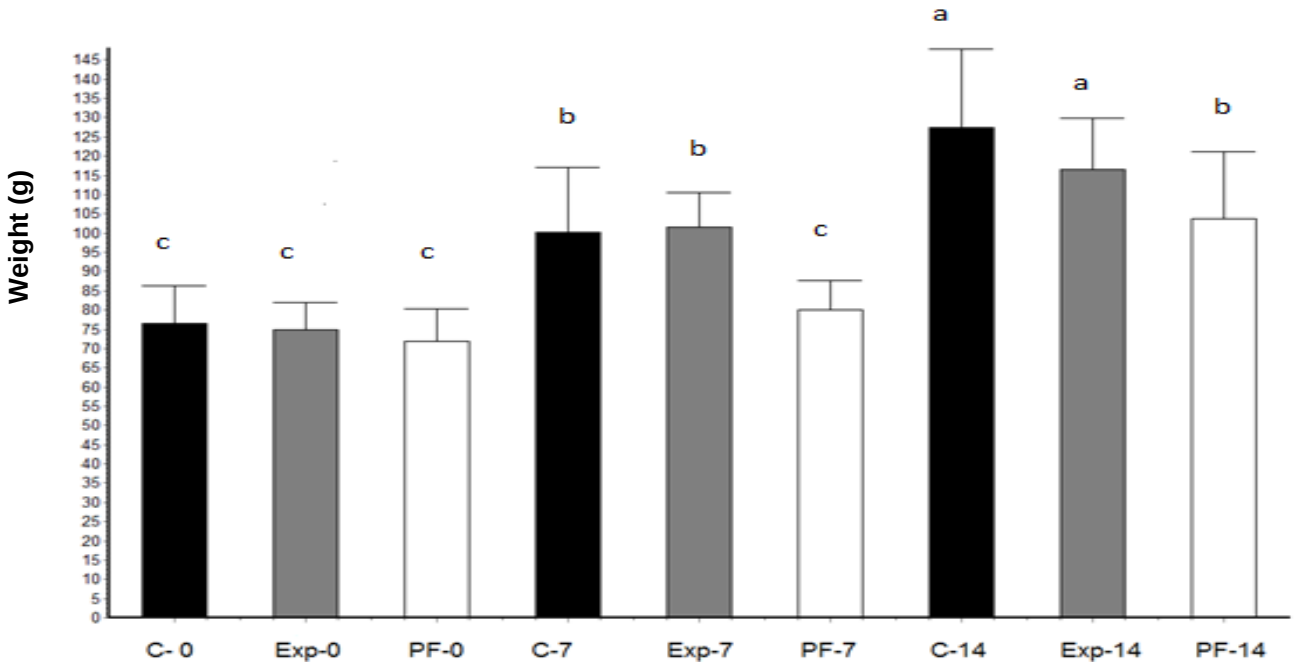

Figure 3. Weights of rats in the control group (C), experimental group 2 (liver, Exp), and paired-feeding group (PF) at baseline (0), on day seven, and on day fourteen. Weights with the same letters are not significantly different according to the Tukey test ($p<0.05$).

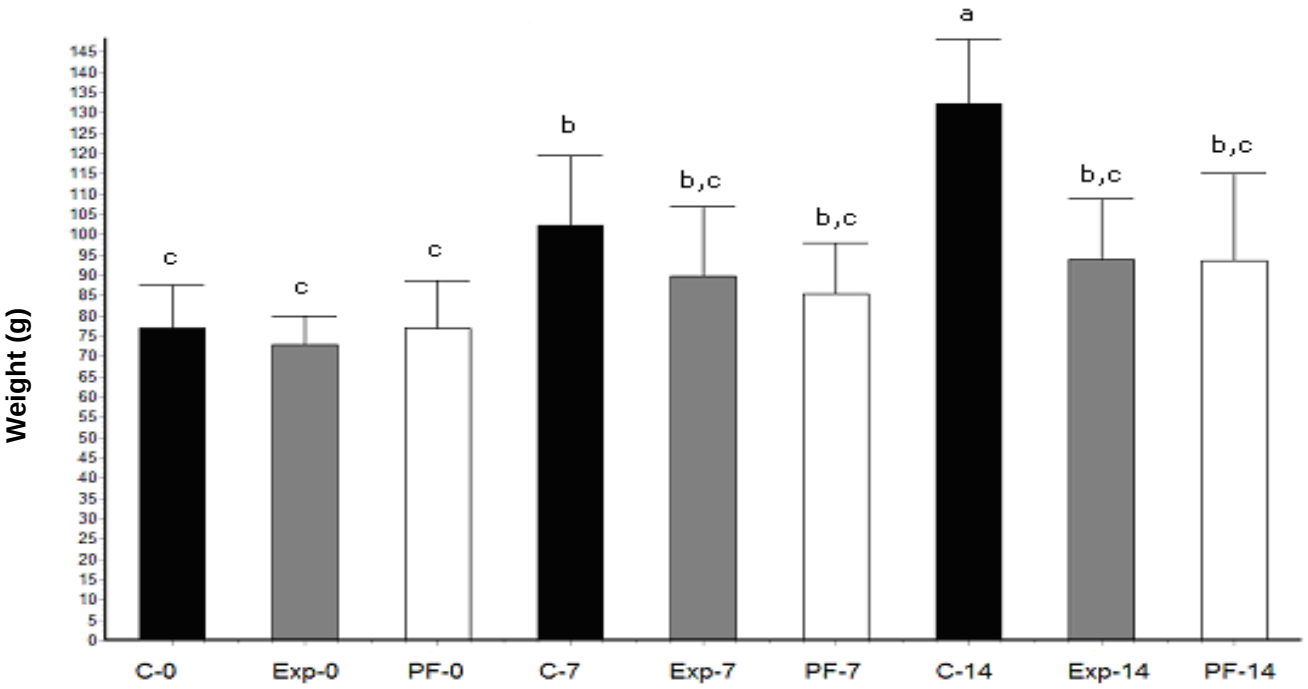

Figure 4. Weights of rats in the control group (C), experimental group 2A (gills, Exp), and paired-feeding group (PF) at baseline (0), on day seven (7), and on day fourteen (14). Weights with the same letters are not significantly different according to the Tukey test ($p<0.05$).

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