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Alternative control of muscle spoilage by a neem cake extract

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Abstract

Interest in the use of antimicrobial packaging systems for muscle and muscle products has increased in recent years to prolong shelf-life, to improve safety, sensory properties, quality of fresh retail meat and to prevent economic loss. The exploration of plant-derived antimicrobials should be an innovative way to find new alternative substances as meat preservatives for antimicrobial packaging. In addition, the use of plant derived antimicrobial products is important as they represent a lower perceived risk to the consumer.

This is the first report dealing with the antimicrobial activity of an ethylacetate ($CH_3COOCH_2CH_3$) neem (*Azadirachta indica* A. Juss) cake extract (NCE) against spoilage bacteria of fresh retail meat (*Escherichia coli*, *Brochothrix thermosphacta, Enterococcus faecalis*, *Carnobacterium* sp., *Lactobacillus curvatus*, *Lactobacillus sakei* and *Leuconostoc* sp.) using the broth macro-dilution method. The percentage of bacterial growth reduction (GR%) varies significantly ($P \geq 0.05$) in function of the concentration of NCE considered (1:10-1:100,000).

The highest percentage of bacterial growth reduction in appropriate liquid medium was obtained at 10 μg of NCE (GR%:79.75±1.53-90.73±1.53). The obtained results showed that the NCE has a broad range of antibacterial activity. The numbers of viable bacterial cells never significantly ($p \leq 0.05$) overcome the inoculums' concentration used to experimentally contaminate meat at each interval considered. NCE should be considered as potential preservative for active packaging of fresh retail meat

KEY WORDS: AZADIRACHTA INDICA, NEEM CAKE, MEAT SPOILAGE, ANTIMICROBIALS, ACTIVE PACKAGING

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Introduction

70 years ago antibiotics changed human health and expectation of life for most of mankind. The discovery of the first antibacterial, penicillin, was reported in 1929, but only in 1938 its use in medicine was speeded up by the war time and the German possible invasion of UK. In 1941 a police constable, a patient in terminal conditions, and a professor of pathology, decided to joint the war effort, opened the Age of Antibiotics. In 1946, a year after the widespread use of antibiotics, several strains of *Streptococcus aureus* evidenced resistance to it. And again, and again, each time a new antibiotic has been developed, the result was the appearance of new resistant bacteria. Use and misuse is heading to the post-antibiotic era. However, if so far the efficacy of current antibiotics limited the research in other fields and other natural substances, natural like penicillin but with different activity and efficacy, are just waiting to be discovered in Nature's arsenal.

Meanwhile, international and impressive bacterial alarms are continuous, in particular in food contamination. In 2011 in Germany a serious food poisoning, caused by a novel resistant strain of the common *E. coli* caused in one month the dead of at least 45 deaths and several thousand hospitalization, not considering a diplomatic affair, because initially Germany blamed Spanish cucumbers of the infection. The strain, not only was multi-resistant, but produced a dangerous toxin, causing bloody diarrhoea and haemolytic-uremic syndrome. As a matter of fact, need for controls is increased by alarms of contaminated food, as well as the necessity to develop a new strategy to avoid the bacteria out of control worldwide consequences.

Fresh retail meat, especially minced meat, is one of the most perishable foods due to its biological composition. The consumer's increased demand for high quality, convenience, safety, fresh appearance and an extended shelf life in fresh meat and meat products, required the development of alternative preservation technologies such as high hydrostatic pressure, superchilling, natural biopreservatives, active and intelligent packaging. Nowadays numerous types of food packaging in combination with different storage techniques can be used in order to extend the shelf-life of meat (1-4). One of the key point during storage is the preservation of the meat from microbial spoilage and contamination/proliferation of pathogenic microorganisms.

Microbial contamination can accelerate major public health risks and economic loss in terms of food poisoning and meat spoilage. Recent food-borne microbial outbreaks are driving a search for innovative ways to inhibit microbial growth in the foods while maintaining quality, freshness,and safety.

Furthermore, the increasing of microorganisms' antimicrobial resistance, coupled with the resultant social and economic implications, causes a constant striving to produce safer feed and food as to develop new natural antimicrobial agents.

Natural compounds, such as essential oils, chitosan, nisin and lysozyme, have been investigated to replace chemical preservatives. Storage life is extended and safety is increased by using natural or controlled microflora, of which lactic acid bacteria (LAB) and their antimicrobial products such as lactic acid and bacteriocins, have been studied extensively (5-7). Various spices and essential oils have preservative properties and have been used to extend the storage life of meat products (8,9). Antimicrobial packaging is a form of active packaging. It interacts with the product or the headspace between the package and the food system, to reduce, inhibit or retard the growth of microorganisms that may be present in the packed food.

The exploration of plant-derived antimicrobials is an innovative way to find new alternative substances as meat preservatives for antimicrobial packaging. In addition, the use of plant derived antimicrobial products is important as they represent a lower perceived risk to the consumer.

This report deals with the antimicrobial activity of an ethylacetate ($CH_3COOCH_2CH_3$) neem (*Azadirachta indica*) cake extract (NCE) against spoilage bacteria of fresh retail meat (*Escherichia coli*, *Brochothrix thermosphacta*, *Enterococcus faecalis*, *Carnobacterium* sp., *Lactobacillus curvatus*, *Lactobacillus sakei* and *Leuconostoc* sp.) evaluated by the broth macro-dilution method.

Methods

A commercial deoiled neem cake produced by NEEM GREEN (www.greeneem.com, Virudhunagar, India) was used as test starting material. Neem cake extract (NCE) was obtained by direct extraction of the dried neem cake with ethylacetate (1:10 wt/v) at room temperature for 24 h. Thereafter the resulted NCE was dried and kept ad -4 °C until further uses. Total composition of the NCE was checked by High Performance Thin Layer Chromatography (HPTLC) and HPLC (High Performance Liquid Chromatography (7).

The meat spoilage bacteria: *Escherichia coli*, *Brochothrix thermosphacta*, *Carnobacterium* sp., *Lactobacillus curvatus*, *Lactobacillus sakei* and *Leuconostoc* sp. were considered in the experiment. These bacterial strains were isolated in a previous study (8) and then maintained in Microbank™ vials at -70°C. The antimicrobial activity of NCE (1mg/1ml Tween 80™) was evaluated as percent of growth reduction in appropriate liquid medium using broth macrodilution method at several NCE concentration (1:10-1:100,000).

The percentage of bacterial growth reduction (GR%) was estimated using as reference the control treatment (T = without extract) as:

$$GR\% = \frac{C - T}{Cx100}$$

Minced meat examined for any bacterial contamination was experimentally inoculated with the bacteria considered in the experiment. Three 10 g samples, taken in aseptic conditions after surface sterilization by U.V. light (2 min for each side of the sample) in biohazard hood at 50 cm distance, were placed in stomacher bags and inoculated with a single strain (ca 10^4 cfu/mL).

The samples were then homogenized in a stomacher (Lab Blender 400, Servard, London, UK) with sterile peptonate water (0.1% peptone and 0.85% NaCl in a 1/10 ratio) for 2 min at room temperature to ensure proper distribution of the bacteria. NCE (10 μg) was added to each inoculated bags. Sterile distilled water and ciprofloxacin were also used as controls. To attain uniform distribution of the added compounds, treated meat samples were further homogenized in a stomacher (Lab Blender 400), as previously described. All stomacher bags with samples from all treatments were sealed under vacuum (Multivac, Type A300/16, Wolfertschenden, Germany) and stored at 10 °C for 12 days, simulating an abusive refrigerated storage.

Numbers of bacterial cells were converted to log cfu and subjected to statistical analyses. Three replicates were considered. Detection of bacteria from experimentally treated meat samples were carried out using microbiological techniques at two day intervals up to the 12th day of refrigerated storage.

The presence of viable cells of all microorganisms tested were checked in beef meat samples experimentally inoculated as previously reported and treated with NCE, distilled water and Tween® 80 at 2, 4, 6. 8. 10, 12 days after treatments.

The results were recorded as means ±SD of the duplicate experiment. Differences between means of data were compared by least significant difference (LSD) calculated using the Statistical Analysis System (S.A.S., Institute, Inc. Cary, NC, USA).

Results

The bacterial growth percentage reduction varies significantly (P ≥ 0.05) in function of the concentration of NCE considered. The highest percentage of bacterial growth reduction in appropriate liquid medium was obtained at 10 μg NCE concentration. The range of bacterial growth reduction (%) at different NCE concentrations, determined in liquid medium with or without NCE, is reported in Table 1.

The bacteria experimentally inoculated in vacuum-packed minced meat treated with NCE were detected at different day as reported in Table 2.

The numbers of viable bacterial cells never significantly ($p \leq 0.05$) overcome the inoculums' concentration used to experimentally contaminate meat at each interval considered (Table 2).

Discussion

Antimicrobial packaging can play an important role in reducing the risk of microbial contamination as well as extending the shelf-life of fresh retail meat. Currently, development is limited due to availability of antimicrobials and new polymer materials, regulatory concerns, and appropriate testing methods. The need for new antimicrobials with wide spectrum of activity and low toxicity is increasing. Research and development of plant derived antimicrobial for food packaging is expected to grow in the next decade. The obtained results showed that the NCE has a broad range of antibacterial activity. It acts as bacteriostatic and bactericidal and it counteracts either gram-positive and gram-negative bacteria or facultative aerobic-anaerobic bacteria in experimental meat system. It should be considered as potential preservative for antimicrobial packaging. Further investigations are in progress. They will focus on the use of biologically active plant derived antimicrobial fitocomplex bound to polymers.

These results are in accordance with the antibacterial activity of neem, that is well known also by use of wings as chewing sticks for dental care, nowadays very popular also in industrialized countries (11). However this is the first report concerning a specific utilization of neem cake as antimicrobial for storage products. The potentialities of neem cake are enormous considering low cost, available quantities and sustainability of the raw material although a lot of work is still necessary for its validation and chemical composition determination.

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Bacteria	Growth Reduction (GR %) Treatment			
	NCE (10 µg)	NCE (1 µg)	NCE (0,1 µg)	NCE (0.001 µg)
Escherichia coli *gc1	79.75±1.53d	30.61±1.00bc	27.67±1.33b	30.811±2.08a
Brochothrix thermosphacta gc2	80.61±1.15d	59.70±1.00bc	37.58±1.33ab	34.86±1.00a
Enterococcus faecalis gc3	90.61±1.15d	69.70±1.00bc	67.58±1.33ab	64.86±1.00a
Carnobacterium sp. gc4	88.90±1.00d	68.79±1.00abc	69.60±0.00ab	66.68±1.20a
Lactobacillus curvatus gc4	90.73±1.53d	69.70±2.08bc	68.69±2.00b	62.83±1.73a
Lactobacillus sakei gc4	89.81±1.00d	69.61±0.58abc	69.57±0.00ab	67.58±0.89a
Leuconostoc sp. gc4	89.67±0.29d	66.56±1.00bc	68.45±0.58ab	61.89±0.58a

Table 1. Bacterial growth reduction (%) at 24h in liquid medium with different concentrations of NCE using as reference the control treatment (without NCE).

NCE = neem cake ethylacetate extract; Values expressed as mean + Standard Deviation of two experiments (three repetitions for each experiment). Mean values with different letter in the column are significantly different (P < 0.05).
*Growth conditions: gc1LB liquid 48 h 37°C; gc2 STAA liquid 48 h 37°C; gc3 Slanetz e Bartley liquid 48 h 37°C; gc4MRS liquid 48 h 35°C.

Bacteria	Bacterial counts (log_{10} CFU/ml) Storage days						
	0	2	4	6	8	10	12
Escherichia coli[1]	6.87±0.5a	6.90±0.5a	6.93±0.5a	6.91±1.2a	6.90±1.5a	0.00	0.00
Brochothrix thermosphacta	7.33±1.0a	7.30±0.0a	7.28±1.0a	0.00	0.00	0.00	0.00
Enterococcus faecalis	7.58±1.0a	7.80±0.0a	7.38±1.0a	0.00	0.00	0.00	0.00
Carnobacterium sp.	7.69±2.0a	7.75±0.5a	7.71±0.0a	0.00	0.00	0.00	0.00
Lactobacillus curvatus	6.95±1.0a	7.30±0.5a	7.21±1.9a	0.00	0.00	0.00	0.00
Lactobacillus sakei	7.20±0.0a	7.49±0.0a	7.28±1.0a	0.00	0.00	0.00	0.00
Leuconostoc sp.	6.97±1.5a	7.11±0.0a	7.20±0.5a	0.00	0.00	0.00	0.00

Table 2. Bacteria counts at 2 days intervals up to the 12th day of refrigerated storage to test the antibacterial activity in experimentally contaminated and treated with NCE (10 µg) beef meat

The numbers of viable bacterial cells never significantly ($p \leq 0.05$) overcome the inoculums' concentration used to experimentally contaminate meat at each interval considered (Table 2).