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*Full Length Research Paper*

# Antiproliferative and antibacterial activity evaluation of red microalgae *Rhodosorus marinus*

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**Antiproliferative activity of three extracts obtained from red microalgae *Rhodosorus marinus* was evaluated against cervical (HeLa), colon (HCT 116), lung (A549), prostate (22Rv-1) and breast (HCC38 and MDA-MB-231) cancer cell lines. Antibacterial activity of these extracts was also tested against *Salmonella choleraesuis*, *Listeria monocytogenes* and *Staphylococcus aureus*. All extracts were obtained from lyophilized biomass of red microalgae. Extract A was obtained using 40% ammonium sulfate precipitation and gel filtration chromatography with G-25 sephadex. Extract B was subjected to a similar process, but 60% ammonium sulfate precipitation was used. Extract C was obtained by methanol extraction and hydrophobicity chromatography using amberlite XAD-2. Protein concentration was determined in two extracts and total phenols in one extract, using Bradford and Folin techniques. Antiproliferative activity was evaluated at extract concentrations ranging from 0.125 to 1 mg/ml, using the spectrophotometric technique MTT (3 - (4,5 - dimetiltiazolyl - 2) - 2,5 - diphenyltetrazolium bromide). The antibacterial activity was evaluated by the impregnated disk test. Extract C showed antiproliferative activity against almost all cancer cell lines with an $IC_{50}$ of 0.5 (HCT 116), 0.8 (HeLa), 0.9 (MDA-MB-231), 0.1 (HCC38), and 0.4 (22Rv-1) mg/ml, whereas none of the tested extracts showed antibacterial activity under experimental conditions.**

**Key words:** *Rhodosorus marinus*, red microalgae, antibacterial activity, antiproliferative activity, methanolic extract.

## INTRODUCTION

Infectious diseases and cancer are important public health problems worldwide. Both types of diseases are different, but share a common characteristic: uncontrolled growth of a cell type (Alberts et al., 2002; Bhunia, 2008). There are drugs whose function is to prevent the growth of harmful cells; antibiotics are used to control bacterial

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populations while antiproliferatives are used in the control of cancer cells growth (Alexander and Hong, 2008; Chang et al., 2003; Granier et al., 2011).

Nowadays, several pathogenic bacteria have shown an increase in antibacterial resistance against some antibiotics commonly used in therapy (Whichard et al., 2007). A similar situation has been observed in cancer treatments, where some antiproliferative drugs gradually decrease their function against cancerous cells (Gottesman, 2002). Drug resistance might be addressed by different strategies, such as prescribed treatment compliance, alternated treatments with different drugs, and the search for new antibacterial and antiproliferative drugs.

A very common source of antibacterial and antiproliferative drugs is vegetable organisms (Aneiros and Garateix, 2004). Plants and other marine organisms have a secondary metabolism, from which many molecules obtained have shown biological activity over several organisms (Saunders, 2009). Actually, this secondary metabolism is also helpful for different activities in the producer organisms, including defense against other organisms and adaptations to special environmental characteristics. Secondary metabolism is also present in marine organisms, but they have been only briefly studied (Jha and Zi-rong, 2004).

Based on the fact that immobile marine organisms inhabit densely populated environments surrounded by predatory mobile organisms, they need to produce inhibitory substances which are released into the marine environment in order to keep away mobile predators (Aneiros and Garateix, 2004; Debbab et al., 2010; Li and Vederas, 2009). However, a high concentration of the released metabolites is required to minimize the dilution effect in the aqueous marine medium. In this way, marine algae among other organisms might be good candidates for searching bioactive molecules capable of inhibiting cell multiplication in the predators and/or competitors.

The presence of pigments other than chlorophylls that allow light absorption in depths where chlorophyll cannot be absorbed is a common characteristic of red algae. Absorbed light is transferred to chlorophyll and, by several reactions, converted into chemical energy that is used by the algae (Eroglu et al., 2013; Lee, 2008). Additional steps required for light absorption before chlorophyll participation include several reactions and the subsequent production of metabolites (secondary metabolism) that might possess biological activities of interest.

Several types of biological activities have been reported in some red seaweed and microalgae species. A *Gracilaria verrucosa* ethanolic extract and its fractions, for example, obtained with ether and ethyl acetate and evaluated for antioxidant activity using the DPPH method, showed significant antioxidant activity *in vitro* tested (Abou and Shalaby, 2009). In addition, an aqueous extract of *G. corticota* showed cytotoxic activity against leukemic cell lines Jurkat and Molt-4 at concentrations of 9.336 and 9.726 µg/ml, respectively (Zandi et al., 2010). Moreover, three extracts from *Gelidium amansii* obtained using phosphate buffer, methanol and dimethyl sulphoxide, were evaluated for their activity against Hepa-1 (murine liver cancer), HL-60 (human leukemia) and NIH - 3T3 (murine embryonic fibroblasts) cells lines. All the extracts showed antiproliferative activity against Hepa - 1 and NIH - 3T3, but not against HL - 60 (Chen et al., 2004).

Phycocyanin is an accessory pigment to chlorophyll, which is present in red algae and some other organisms like cyanobacteria (Gantar et al., 2012; Martelli et al., 2014). For example, the antioxidant activity of *Spirulina* has been highly correlated with phycocyanin (Estrada et al., 2001). Phycocyanin obtained from *S. platensis* has already shown to induce apoptosis in murine cancer cells AK-5, through induction of reactive oxygen species and inhibition of cyclooxygenase-2 (COX-2). COX-2 catalyzes arachidonic acid conversion into prostaglandins; and overexpression of prostaglandins stimulates cell proliferation and Bcl-2 inhibition. The protein Bcl-2 inhibits cell apoptosis, Bcl-2 is also inhibited by phycocyanin (Pardhasaradhi et al., 2003).

The flavonoids compounds are present in phenolic extracts. The flavonoids are compounds that have shown antibacterial and antiproliferative activities in several organisms (Liu et al., 2013; Talib et al., 2012). According to previous information, protein and phenolic extract could be a potential source of bioactive compounds.

The red microalga *Rhodosorus marinus* which belongs to Rodophyta division and Rhodophyceae class is easy to culture in the laboratory under controlled laboratory conditions such as ambient temperature and synthetic culture medium elaborated with seawater. This microalga presents the majority of red seaweeds metabolic characteristics, and, being a unicellular organism, it is an optimal model to study red algae for biological evaluations.

The aim of this research work was to evaluate protein and phenolic extracts obtained from *R. marinus* for antiproliferative activity against cervical, colon, lung, prostate, and breast cancer cell lines, as well as for antibacterial activity against *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella choleraesuis*.

## MATERIALS AND METHODS

### Biomass obtention from microalgae

Red microalga *R. marinus* strain UTEX 1723 was acquired from Texas University collection. Microalgae were cultured for 60 days in modified Erdschreiber medium ($NaNO_3$ 0.02%, $NaH_2PO_4$ 0.003%, $FeCl_3$ 1.16 mg/L, $MnCl_2$ 0.5 mg/L, $ZnCl_2$ 0.06 mg/L, $CoCl_2$ 0.024 mg/L, $Na_2MoO_4$ 0.048 mg/L, cyanocobalamine 0.01 mg/L and thiamine 0.2 mg/L, dissolved in sterilized seawater (salinity of 35 g/L), with continuous air bubbling, 12/12 h dark/light cycles, and a temperature of 25 ± 2°C. The culture was then centrifuged at 3600

x *g*, 4°C, during 10 min (Eppendorf centrifuge 5804 R); supernatant was discarded and the pellet was washed twice with distilled water and then lyophilized at - 90°C, low pressure during 70 ± 5 h (Labconco Freeze zone 6 plus) and dried biomass was collected for further analyzes. Unless otherwise stated, all chemicals used were purchased from Sigma Aldrich Co.

**Protein extracts**

Protein extracts from *R. marinus* (Extracts A and B) were obtained as previously reported by Básaca-Loya et al. (2009). This method was slightly modified as follows. Lyophilized biomass (2 g) was re-suspended in 50 ml of PBS (0.01 M $Na_2HPO_4$, 0.1 M NaCl, pH 7.0), and the *R. marinus* suspension was maintained during 30 min at -80°C, and then it was manually macerated during 5 min using mortar and pestle. The macerated solution was sonicated during 30 min using Cole Parmer 8890 equipment, and centrifuged at 5000 × *g*, during 15 min, at 4°C. The supernatant was filtered through a Whatman # 1 filter paper, saturated with 40% $(NH_4)_2SO_4$, and maintained under gentle agitation during 3 h, at 4°C. After that, the solution was centrifuged as previously described, the pellet was collected (Extract A), and the supernatant was saturated until 60% $(NH_4)_2SO_4$ was reached. This last solution was maintained under gentle agitation during 12 h, at 4°C, and centrifuged again under the same conditions, the supernatant was discarded, and the pellet was collected (Extract B) for further use.

Both extracts were fractionated by gel filtration chromatography, using sephadex G-25 as the stationary phase and PBS as the eluent, at 1 ml/min flow rate. Elution of the components of the extracts was monitored at 280 nm (Beckman coulter spectrophotometer DU 530). After elution, each extract was collected and dialyzed. Dialysis process was performed using a membrane tubing (Spectrum 132655, MWCO 6 - 8 kDa) that was sealed and placed in 5 successive water baths containing deionized water at 4°C and gentle stirring at time 0, 4, 16, 20 and 24 h, respectively; then, both extracts were lyophilized under the conditions previously described.

**Protein quantification**

Protein quantification was performed following the method described by Bradford (Bradford, 1976). A standard curve was prepared using bovine serum albumin in concentrations from 62.5 to 2 mg/mL. Color development was measured at 595 nm, 5 min after reagent addition. Additionally, SDS-PAGE chromatography was performed, as described by Grabski and Burgess (2010).

**Phenolic extract**

Phenolic extract (Extract C) was obtained according to the techniques previously described (Martos et al., 2000), with the following variations: lyophilized *R. marinus* biomass (2 g) was suspended in 50 ml of acid water (deionized water, pH 2.0). The suspension rested at -80°C, during 30 min; then it was manually macerated, during 5 min. After a 30 min-period of sonication, the solution was centrifuged at 5000 × *g*, at 4°C, during 10 min, and the pellet was discarded. The supernatant was passed through an Amberlite XAD-2 (Fluka, pore size 9 nm, particle size 0.3 to 1.2 mm) column (25 × 2 cm).

Phenolic compounds remained in the column, while sugars and other polar compounds eluted with the aqueous solvent. The column was washed with acid water (pH 2.0, 125 ml) and subsequently with deionized water (125 ml). The whole phenolic fraction was finally eluted with methanol (250 ml) and taken to dryness under reduced pressure (145 Torr, 45°C). Finally, the extract was suspended in 10 ml of water and lyophilized under the same conditions described above.

**Total phenolic compound quantification**

Total phenolic compounds content was measured using Folin - Ciocalteu´s phenol reagent (Waterhouse, 2003). Fifty microliters of extract were mixed with 3 mL of water and 250 µL of 1 N Folin reagent. After 5 min, 750 µL of 20% sodium bicarbonate and 950 µL of water were added and maintained for 30 min at room temperature. Finally, absorbance was measured at 765 nm. A standard curve was obtained using gallic acid (G7384) in concentrations from 80 to 400 µg/ml.

**Antiproliferative evaluation**

HeLa (ATCC CCL2), HCT 116 (ATCC CCL247), A549 (ATCC CCL185), and ARPE 19 (ATCC CRL2302) cells were cultured in MEM medium (D7777) supplemented with 10% bovine fetal serum (BF6178). For MDA-MB-231 cells (HTB 26), MEM medium was also used, but 15% bovine fetal serum, 1% L-glutamine (G6392), and 1% non-essential aminoacid solution (M7145), were added. Finally, for HCC-38 cells (ATCC CRL2314) and 22Rv-1 (ATCC CRL2505), RPMI medium (R4130) supplemented with 10% bovine fetal serum and 1% L-glutamine, was used. All cell cultures were maintained at 36 ± 1°C, 5% $CO_2$ atmosphere and 85% of moisture, until 95% of confluence was reached. Cells were detached with trypsin-EDTA solution (T4049) and a cellular suspension of $2x10^5$ cells/ml was prepared.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay (ATCC 30-1010K) was performed as previously described (Mhadhebi et al., 2011). Briefly, 50 µL of cellular suspension were placed in microtiter plate wells (in triplicates) and incubated overnight at 36 ± 1°C, 5% $CO_2$ atmosphere and 85% of moisture. After that, 50 µL of extract, dissolved in the same medium used for culture, was added. The tested concentrations included serial dilutions from 1 to 0.125 mg/ml of each extract in the appropriate culture medium. Cell cultures in microtiter plates were incubated during 48 ± 1 h under the same previously described conditions. Then, 10 µL of MTT solution were added to each well and incubated for another 4 h-period. Finally, 150 µL of acidic isopropanol (0.04-0.1 N HCl in absolute isopropanol) were added to dissolve formazan crystals. The absorbance was measured at 570 nm. Fresh medium and 25 mmol cis-diammineplatinum (II) dichloride (P4394) solutions were used as experimental controls. The experiment was repeated three times.

**Antibacterial evaluation**

Antibacterial activity was determined using the disk diffusion assay (Ravaei et al., 2013). Bacterial strains used for the experiment were *S. choleraesuis* (ATCC 14028), *L. monocytogenes* (ATCC 19111), and *S. aureus* (ATCC 25923). All the bacteria were grown in tryptic soy broth (BD 257107), 36 ± 1°C, for 24 h. Blank disks (5 mm diameter, 1 mm thickness) were impregnated with 10 µL of extract and let to dry; then, they were impregnated again with the same amount of extract. The concentration used of each extract was 1 mg/ml. Blank disks, impregnated with PBS and penicillin - streptomycin solution (P0781), were used as negative and positive control, respectively. Twenty milliliters of molten Mueller-Hinton agar (BD 221275) were poured into 8 cm sterile Petri dishes and let to solidify. One-hundred microliters of the overnight broth culture of each bacterial strain (previously adjusted to 0.5 McFarland turbidity), were spread on the plates. A disk impregnated with the test extract and two control disks were placed on the surface of the

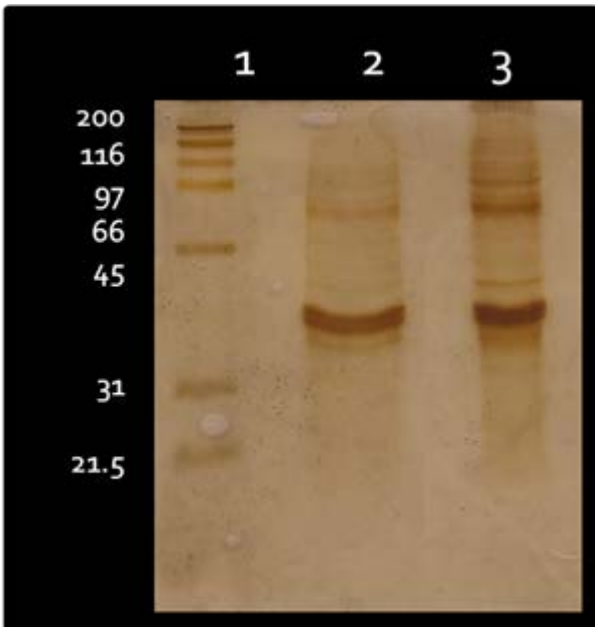


**Figure 1.** Electrophoretic profile obtained for protein extracts using SDS-PAGE and silver stain. Molecular weights are expressed in kDa. Lane 1, molecular weight marker; lane 2, extract obtained with 40% ammonium sulfate precipitation; and lane 3, extract obtained with 60% ammonium sulfate precipitation.

agar and incubated at 36 ± 1°C, for 24 h. The experiment was carried out in triplicates. An inhibition zone around the disks was interpreted as a positive qualitative antibacterial activity.

#### Statistical analysis

Cell proliferation graphs were obtained using Graphpad prism version 3.02. Tukey means comparison test (95%) was used to determine difference between cells proliferation under different extracts concentrations. For $IC_{50}$ a linear regression was performed. Means comparisons and linear regression were analyzed using NCSS 6.0 software.

## RESULTS

### Quantification of extracts

Two calibration curves were required for the quantification of protein and phenolic extracts. For protein extract, a calibration curve was made with bovine serum albumin obtaining the following equation: $A = 0.7129C - 0.0042$ ($R^2 = 0.9982$); where, $A$ represents absorbance and $C$ the test extracts concentration. Using the previous equation, protein concentration in the extract A was 0.697 mg/ml, whereas for extract B, the protein concentration was 1.103 mg/ml. The electrophoretic profiles for both protein extracts obtained by SDS-PAGE are shown in Figure 1.

For the quantification of total phenolic compounds in extract C, the calibration curve was made using gallic acid, obtaining the following equation: $A = 0.9621C + 0.0006$ ($R^2 = 0.9984$). According to this equation, the concentration of total phenolic compounds in the extract C was 7.67 mg equivalents of gallic acid / g in dry weight basis.

### Antiproliferative evaluation

Two different effects were observed in the cancerous cell lines when exposed to the test extracts. In Figure 2, these two different effects could be observed; extract C was able to inhibit CCL247 cell proliferation showing a dose-response type of relationship, while HTB26 cell line was not inhibited by the extract B. The statistical analysis confirmed previous observations.

The $IC_{50}$ value, defined as the extract concentration that resulted in 50% of cell growth inhibition, was calculated for each extract versus each cell line (Table 1). A relationship between $IC_{50}$ value and proliferation response was observed in our experiment. When an inhibition of cell growth was obtained the $IC_{50}$ parameter was a positive value, whereas in non-effect or slightly promotion effect, a negative $IC_{50}$ value was obtained.

Table 1 shows all the $IC_{50}$ calculated for the effect of the three extracts against each tested cell line.

### Antibacterial evaluation

No antibacterial effect was observed when bacterial species *S. aureus*, *L. monocytogenes* and *S. choleraesuis* were exposed to the extracts, under previously described conditions.

## DISCUSSION

Electrophoretic patterns (Figure 1) of extracts A and B were quite similar, although extract B (1.103 mg/ml) had almost twice protein compared to extract A (0.697 mg/ml). The intensity of the bands in electrophoretic gels was consistent with the protein concentration. A previous report (Básaca-Loya et al., 2009), proposed that extract A included a set of proteins different from the ones obtained in extract B. According to this previous report, just extract B contained phycobiliproteins, and extract A was just a set of proteins different of phycobiliproteins that could be discarded. However, electrophoretic profiles showed similar patterns with apparently higher protein concentration in extract B. Previous observation was confirmed using Bradford to determine protein content and, in this way, inferring that both extracts might include the same set of proteins, just with different concentration, was possible. No further characterization was performed because neither extract A nor extract B showed biological activity under tested conditions.

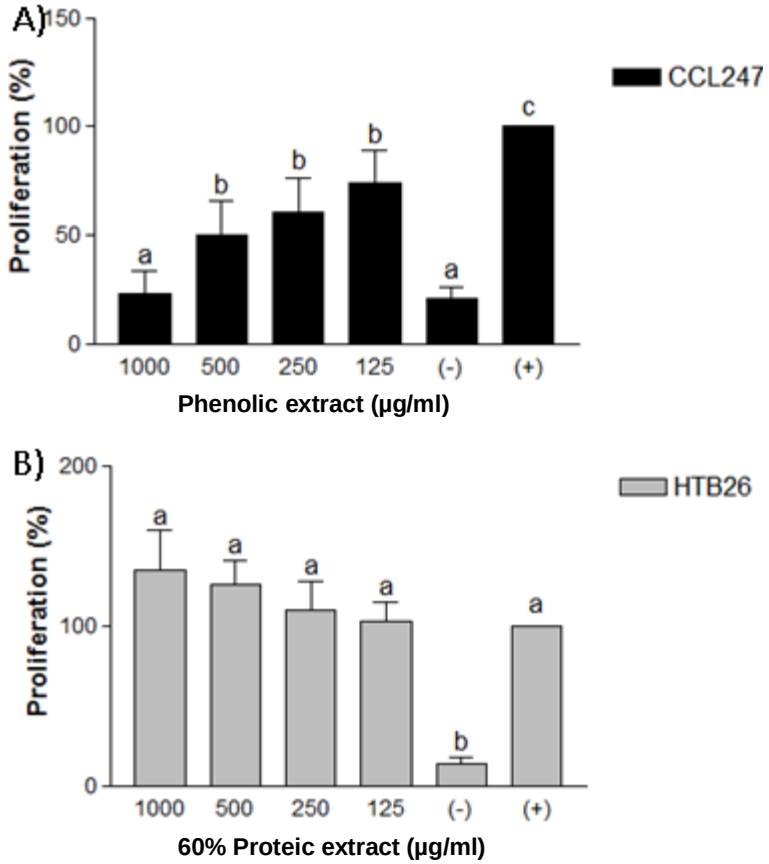


**Figure 2.** Different effects of tested extracts in cancer cells proliferation. A) an inhibition in growth of CCL247 by the concentration effect of phenolic extract. B) non effect in HTB26 proliferation due to different concentrations of 60% protein extract addition. Different literal in bars means a statistical difference ($p<0.05$). (-) 25 mmol cis-diammineplatinum (II) dichloride, a commercial antiproliferative drug and (+) fresh culture medium.

**Table 1.** $IC_{50}$ value in mg/mL (the total extract concentration that resulted in 50% of cell growth inhibition) for the three tested extracts and Cis-Pt (commercial antiproliferative drug used as control) against the different cell lines. ND means not detected, because no relationship could be observed between extract concentration and cell proliferation.

| Cell line | Extract A | Extract B | Extract C | Cis-Pt |
|---|---|---|---|---|
| CCL2 (HeLa) | ND | -1.0921 | 0.8010 | 0.0080 |
| CCL247 (HCT 116) | ND | ND | 0.5031 | 0.0073 |
| HTB26 (MDA-MB-231) | ND | -1.4006 | 0.9401 | 0.0068 |
| CRL2314 (HCC38) | -0.2175 | -1.1862 | 0.1776 | 0.0127 |
| CCL185 (A549) | -0.1214 | -0.6520 | ND | 0.0096 |
| CRL2505 (22Rv-1) | -0.2436 | -0.5836 | 0.4088 | 0.0079 |
| CRL2302 (ARPE19) | -1.1623 | -1.1201 | ND | ND |

Reports of total protein content in red microalgae are not common; however, when phycocyanin was obtained from *R. marinus* in a previous work (López, 2011); a total protein concentration of 0.478 mg/mL was reported for the 60% ammonium sulfate precipitation fraction (similar to extract B). In our work, 1.103 mg/ml were obtained, which is a higher concentration than 0.478 mg/ml. This could be explained by the modifications proposed to the original extraction technique.

Total phenolic compounds have been reported for microalgae. In a previous study for *S. maxima,* 4.51 mg/g dry weight in phenolics (gallic acid equivalents) were

reported (El-baky et al., 2009). This value is higher than that obtained in the present work for *R. marinus*. Both species, *S. maxima* and *R. marinus,* are unicellular organisms, but the auxiliary pigments to chlorophyll differ from each other, since *R. marinus* is considered a red microalga and *S. maxima* is classified as a cyanobacterium. These considerations might in part explain the differences in the content of phenolic compounds.

In addition, total phenolic compounds content has been studied in some red macroalgae when biological activity was studied (Echavarria et al., 2009), with values for phenolics ranging from 0.15 to 1.98 mg equivalents of gallic acid / g in a dry weight basis, these values being lower than those obtained in present research work.

Although red seaweeds have been studied in a very limited way as a source of antiproliferative compounds, there are still some studies where antioxidant activity of phenolic extracts has been tested, and strong antioxidant activity was observed in *G. verrucosa* and some other red seaweed from the Gulf of Thailand (Abou and Shalaby, 2009; Boonchum et al., 2011). It was proposed that some antioxidant molecules, such as vitamins, enzymes, isolated from marine organisms, including microalgae, could also show antiproliferative activity (Debbab et al., 2010).

The main phycobiliprotein present in *R. marinus,* phycoerythrin, was already isolated and tested for its antiproliferative activity against HeLa cells (Rascon-Durán, 2009). A negative response was obtained; no antiproliferative activity of phycoerythrin against HeLa cells was verified. These results are similar to those obtained in the present study, where both biliprotein extracts (A and B), which include phycoerythrin and other phycobiliproteins, showed no antiproliferative activity against all cancer cell lines tested. Phycocyanin (phycobiliprotein also present in *R. marinus*) was also tested against murine cancer cells, with phycocyanin in lower concentrations than concentrations used in this work showing antiproliferative activity (Pardhasaradhi et al., 2003). This difference in the results could be due to the fact that all cancer cells used in our work were from human isolates; this seems to be important, since species specificity in antiproliferative activity was previously referred (Alberts et al., 2002).

As previously mentioned, few studies aimed on antiproliferative activity evaluation using red seaweeds have been reported. One of these reports evaluated an aqueous extract from red macroalga *G. corticota* against human leukemic cells using higher concentrations than concentrations used in present work showing the better inhibition activity at 9.3 mg/ml extract concentration (Zandi et al., 2010). Actually, these authors found a small antiproliferative activity but $IC_{50}$ was not reported.

The potential of red macroalgae as a source of antiproliferative compounds needs to be studied in a broader way, since some investigations have shown an activity against normal cells. The aqueous and methanolic extracts obtained from *Ge. amansii* showed antiproliferative activity against murine hepatoma cells, but also against murine embrionary fibroblasts (Chen et al., 2004).

On the other hand, the phenolic extract (Extract C), obtained from *R. marinus* showed antiproliferative activity against CCL2, HTB26, CCL247, CRL2505 and CRL2314 (cancer cells lines), but not against CRL2302 (non-cancerous cell line) and CCL 185 (also a cancer cell line) (Table 1). It is important to consider that a positive value in $IC_{50}$ indicates the concentration required to inhibit the proliferation of 50% of exposed cells, whereas a negative value indicates a non-effect in antiproliferative activity or a slightly promoter effect. As in this case, when a screening study is performed in order to find a possible biological activity, tested values with antiproliferative activity needs to be smaller than 1 mg/ml total extract concentration, higher concentrations could affect all the exposed cells (cancer and normal cells) by osmotic pressure of the extract (Picot et al., 2006). Due to this reason, the value obtained for CRL2302 ($IC_{50}$ = 3.9321 mg/ml) is not considered as having an important biological activity. In other words, the required amount of phenolic extract in order to reach at least a 50% antiproliferative effect in CRL2302 would be almost 4 mg/ml, and usually a commercial antiproliferative drug is administrated in concentrations smaller than 1 mg/ml. In Table 1 it is possible to observe that $IC_{50}$ obtained for extract C is much bigger than for Cis-Pt (commercial antiproliferative drug used as control), it is important to recall that Cis-Pt is an isolated kind of molecules and the extract is a rich mixture of different kinds of molecule, for this reason an extract could have $IC_{50}$ bigger than isolated molecules (until 1 mg/ml as previously mentioned).

In *Cystoseira crinita*, a brown seaweed, the antiproliferative activity of a methanolic extract of phenolic compounds was obtained by a process quite similar to the one used in this piece of work and a $IC_{50}$ of 58 and 80 µg/ml were reported by Mhadhebi et al. (2011) against colon and breast cancer cell lines, respectively (Mhadhebi et al., 2011). Compounds other than phycobiliproteins might be the responsible for this biological activity.

Based on the above, *R. marinus* could be a good source of new molecules with a potential to be used as antiproliferative agents against some human cancer cells. In terms of the kind of extract tested in this study, the phenolic extract was a better choice than protein extracts. None of the tested extracts showed antibacterial activity, but their antiproliferative potential is promising. The characterization of the phenolic extract and the addition of other test, are needed to perform a more complete description of its activity. For the purpose of this research work, red microalgae have a potential for being further studied as a source of novel molecules with biological

activity.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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### REFERENCES

Abou F, Shalaby EA (2009). Antioxidant Activity of Extract and Semi-Purified Fractions of Marine Red Macroalga, Gracilaria verrucosa. Aust. J. Basic Appl. Sci. 3:3179-3185.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002). Molecular Biology of the Cell, 4th ed. Garland Science, Washington, D.C.

Alexander BM, Hong TS (2008). Rectal Cancer, in: Chabner, B., Lynch, T., Longo, D. (Eds.), Harrison´s Manual of Oncology. McGraw-Hill, Washington, D.C.

Aneiros A, Garateix A (2004). Bioactive peptides from marine sources: pharmacological properties and isolation procedures. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 803:41-53. doi:10.1016/j.jchromb.2003.11.005

Básaca-Loya G, Valdez M, Enríquez-Guevara E, Gutiérrez-Millán L, Burboa M (2009). Extraction and purification of B-phycoerythrin from the red microalga Rhodosorus marinus. Ciencias Mar. 35:359-368.

Bhunia AK (2008). Foodborne Microbial Pathogens. Springer, West Lafayette.

Boonchum W, Peerapornpisal Y, Kanjanapothi D, Pekkoh J, Pumas C, Jamjait U, Amornlerdpison D, Noiraksar T, Vacharapiyasophon P (2011). Antioxidant Activity of some Seaweed from the Gulf of Thailand. Int. J. Agric. Biol. 13:95-99.

Bradford MM (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Chang S, Sievert D, Hageman J, Boulton M, Shah S, Rudrik J, Pupp G, Brown W, Cardo D, Fridkin S (2003). Infection with Vancomycin-Resistant Staphylococcus aureus Containing the vanA Resistance Gene. New Engl. J. Med. 348:1342-1347.

Chen YH, Tu CJ, Wu HT (2004). Growth-inhibitory effects of the red alga Gelidium amansii on cultured cells. Biol. Pharm. Bull. 27:180-184.

Debbab A, Aly AH, Lin WH, Proksch P (2010). Bioactive compounds from marine bacteria and fungi. Microb. Biotechnol. 3:544-563. doi:10.1111/j.1751-7915.2010.00179.x

Echavarria B, Franco A, Martinez A (2009). Evaluación de la actividad antioxidante y determinación del contenido de compuestos fenólicos en extractos de macroalgas del caribe colombiano. Vitae, Rev. la Fac. Química Farm. 16:126-131.

El-baky HH, Baz FK, El-baroty GS (2009). Production of phenolic compounds from Spirulina maxima microalgae and its protective effects. Afr. J. Biotechnol. 8:7059-7067.

Eroglu E, Eggers PK, Winslade M, Smith SM, Raston CL (2013). Enhanced accumulation of microalgal pigments using metal nanoparticle solutions as light filtering devices. Green Chem. 15:3155-3160. doi:10.1039/c3gc41291a

Gantar M, Simović D, Djilas S, Gonzalez WW, Miksovska J (2012). Isolation, characterization and antioxidative activity of C-phycocyanin from Limnothrix sp. strain 37-2-1. J. Biotechnol. 159:21-26. doi:10.1016/j.jbiotec.2012.02.004

Gottesman MM (2002). Mechanisms of cancer drug resistance. Annu. Rev. Med. 53:615-627.

Grabski AC, Burgess RR (2010). Preparation of protein samples for SDS-polyacrylamide gel electrophoresis: procedures and tips. Innovations 13:10-12.

Granier SA, Moubareck C, Colaneri C, Lemire A, Roussel S, Dao TT, Courvalin P, Brisabois A (2011). Antimicrobial resistance of Listeria monocytogenes isolates from food and the environment in France over a 10-year period. Appl. Environ. Microbiol. 77:2788-2790. doi:10.1128/AEM.01381-10

Jha RK, Zi-rong,X (2004). Biomedical compounds from marine organisms. Mar. Drugs 2: 123-146.

Lee RE (2008). Phycology, 4th ed. Cambridge University Press, New York.

Li JW, Vederas JC (2009). Drug discovery and natural products: end of an era or an endless frontier? Science 325:161-165. doi:10.1126/science.1168243

Liu R, Zhang H, Yuan M, Zhou J, Tu Q, Liu JJ, Wang J (2013). Synthesis and biological evaluation of apigenin derivatives as antibacterial and antiproliferative agents. Mol. 18:11496-11511. doi:10.3390/molecules180911496

López A (2011). Purificación de Ficocianina Extraída de la Microalga Roja, Rhodosorus marinus. Universidad de Sonora, México.

Martelli G, Folli C, Visai L, Daglia M, Ferrari D (2014). Thermal stability improvement of blue colorant C-Phycocyanin from Spirulina platensis for food industry applications. Process Biochem. 49:154-159. doi:10.1016/j.procbio.2013.10.008

Martos I, Ferreres F, Tomás-Barberán F (2000). Identification of flavonoid markers for the botanical origin of Eucalyptus honey. J. Agric. Food Chem. 48:1498-1502.

Mhadhebi L, Clary AL, Robert J, Bouraoui A, Victor U, Bordeaux S, Bergonié I, Argonne C, Cedex B (2011). Anti-inflammatory, anti-proliferative and anti-oxidant activities of organic extracts from the Mediterranean seaweed, Cystoseira crinita. Afr. J. Biotechnol. 10:16682-16690. doi:10.5897/AJB11.218

Pardhasaradhi BV, Ali AM, Kumari AL, Reddanna P, Khar A (2003). Phycocyanin-mediated apoptosis in AK-5 tumor cells involves down-regulation of Bcl-2 and generation of ROS. Mol. Cancer Ther. 2:1165-1170.

Picot L, Bordenave S, Didelot S, Fruitier-Arnaudin I, Sannier F, Thorkelsson G, Bergé JP, Guérard F, Chabeaud A, Piot JM (2006). Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. Process Biochem. 41:1217-1222. doi:10.1016/j.procbio.2005.11.024

Rascon-Durán AD (2009). Obtención de B-Ficoeritrina de la microalga roja Rhodosorus marinus y evaluación in vitro de su efecto en la proliferación de células de cáncer cervicouterina (HeLa). Universidad de Sonora, México.

Ravaei A, Heshmati Z, Salehi TZ, Tamai IA, Ghane M, Derakhshan J (2013). Evaluation of Antimicrobial Activity of Three Lactobacillus spp . against Antibiotic Resistance Salmonella typhimurium. Adv. Stud. Biol. 5:61-70.

Saunders C (2009). The anti-proliferative effect of different tomato varieties on the human colon adenocarcinoma cells. Biosci. Horizons. 2:172-179. doi:10.1093/biohorizons/hzp020

Talib WH, Zarga MH, Mahasneh AM (2012). Antiproliferative, antimicrobial and apoptosis inducing effects of compounds isolated from Inula viscosa. Mol. 17:3291-3303. doi:10.3390/molecules17033291

Waterhouse AL (2003). Determination of Total Phenolics. Curr. Prot. Food. Chem. 13:256-258.

Whichard JM, Gay K, Stevenson JE, Joyce KJ, Cooper KL, Omondi M, Medalla F, Jacoby G, Barrett TJ (2007). Human Salmonella and concurrent decreased susceptibility to quinolones and extended-spectrum cephalosporins. Emerg. Infect. Dis. 13:1681-1688.

Zandi K, Tajbakhsh S, Nabipour I, Rastian Z, Yousefi F, Sharafian S, Sartavi K (2010). In vitro antitumor activity of Gracilaria corticata ( a red alga ) against Jurkat and molt-4 human cancer cell lines. Afr. J. Biotechnol. 9:6787-6790.

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*Full Length Research Paper*

# Plasmid profile of multi antibiotic resistant *staphylococcus aureus* isolated from diabetic wounds from patients at Nsukka, South-eastern, Nigeria

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**Multi-drug resistant bacterial strains evolving worldwide has created a great public health problem that needs urgent attention; as such bacteria show resistance to the drug of choice for treatment as well as being resistant to newer and last line antibiotics. In this study, the antibiotic susceptibility, multi antibiotic resistance (MAR) index and plasmid profile of MDR *Staphylococcus aureus* isolated from diabetic wounds of patients presenting at Renaissance hospital Nsukka, Southeast, Nigeria were investigated. Using basic bacteriological and biochemical techniques, *S. aureus* was isolated from all 34 specimens and 19 of these showed multi-drug resistances to most of the commonly prescribed antibiotics in the region, with methicillin and vancomycin inclusive. The 19 MDR isolates were screened for the presence of plasmids as well as calculating the multi-antibiotic resistance (MAR) index. The results show the presence of plasmids in 18 (94.73%) of the specimens; while there was no plasmid in one. The plasmids varied in the range of their molecular sizes and nine different plasmid profile groups were identified ranging between 4946 (bp) to 12130 (bp). For the 19 MDR isolates, the calculated MAR index was greater than 0.2. The findings from this study show that 56% of the isolated *S. aureus* were not susceptible to current antibiotics. This could suggest an imminent change in resistant pattern as observed, particularly in an area already reported as high antibiotic use.**

**Key word:** *Staphylococcus aureus,* susceptibility, antibiotic resistance, plasmids, diabetic wound.

## INTRODUCTION

The challenge of multidrug resistant (MDR) bacterial strains is enormous, particularly in this 21st century. With the threat posed by MDR bacteria, there should be an urgent need to curtail their menace and reverse their imminent spread. Recently, methicillin resistant *Staphylococcus aureus* (MRSA) has emerged worldwide

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as an endemic pathogen. Incidence of MRSA is on the increase globally and at an alarming rate (Mishra et al., 2013). The bacterium is also reported to be resistant to many new drugs as well (Gould et al., 2012). MRSA is the cause of a number of human infections, some of which are fatal, invasive and could lead to toxic conditions (Subedi and Brahmadathan, 2005; Dhanoa et al., 2012). The fact that *S. aureus* infections are found to be prevalent in hospitals, the community has highlighted the need for an immediate action according to Brennan et al. (2012). MRSA is reported to have distinctive phenotypic and genetic features whether community or hospital acquired (Mammina et al., 2012). Evidence have shown that some strains of *S. aureus* are found to be resistant to the last line antibiotics (Park and Lui, 2012; De Vriese and Vandecasteele, 2014) and this has restricted the options available for its treatment in many regions of the world (Subedi and Brahmadathan, 2005). One of such regions is Nigeria (Akinyemi et al., 1997) where antibiotic resistance may be due to spontaneous mutation or acquired through plasmid transfer from other resistant bacteria (Bhaktar et al., 2003). Therefore, in many instances, resistance to antimicrobial agents by Staphylococci is attributed to the presence of plasmids that carry the genetic determinants for resistance (Adeleke et al., 2002). However, other workers have attributed this behaviour to chromosomal mutation (Chibuike et al., 2014). Plasmids have the ability to mediate the production of drug inactivating enzyme such as β lactamases (King et al., 2006; Diep et al., 2008; Esimone et al., 2010). Reports have indicated that plasmid-encoded antibiotic resistance encompasses most classes of antibiotics currently in clinical use (Esimone et al., 2010). Studies have also shown that plasmid profiles are useful in epidemiological surveillance of disease outbreaks and in tracing antibiotic resistance (King et al., 2006; Diep et al., 2008). Different patterns of antibiotic resistance and plasmid profiles among strains of *S. aureus* have been reported using plasmid profile (Bhaktar et al., 2003; Diep et al., 2008). In the present study, we report the isolation of multi-antibiotic resistant *S. aureus* from diabetic wounds of out-patients attending Renaissance Hospital at Nsukka, Nigeria. Their pattern of antibiotic susceptibility was determined in addition to multi antibiotic resistance (MAR) index and plasmid profile.

## MATERIALS AND METHODS

### Bacteria strain

Multi-antibiotic resistant *S. aureus* isolated from wounds of diabetic outpatients attending Renaissance hospital, Nsukka, Enugu State of Southeastern Nigeria were used for this study. A criterion for inclusion in this study was to sample patients who have had these wounds for three months and above; indication that wounds are not healing despite treatment. Informed and written consent were obtained from patients before the start of sampling with the permission of the Renaissance hospital, after the study protocol was approved by the departmental ethical committee. The study protocol was in accordance with good clinical practice and ethical values outlined in the Helsinki Declarations.

*S. aureus* was isolated on mannitol salt agar (MSA) (Oxoid England) and incubated at 37°C for 24 h. Microbial characterization of the bacterial isolates was based on microbiological methods described by Dionigi et al. (2002). Each distinctive morph type of mannitol-fermenting colony was selected from the MSA plate and sub-cultured on blood agar (Zayo Sigma, Germany) at 37°C for 24 h. Incubated cultures on blood agar were screened using method described by Cowan and Steel (2004). *S. aureus* was identified by colony morphology, Gram stain, DNase catalase and coagulase tests as well as fermentation of mannitol.

### Antimicrobial susceptibility testing

The susceptibility of isolates to commonly used antibiotic was determined by the disk diffusion method for *in-vitro* antibiotic susceptibility as described by NCCL (2002), against the following antibiotics; cotrimoxazol (25 µg), cloxacillin (5 µg), erythromycin (5 µg), gentamicin (10 µg), augmentin (30 µg), streptomycin (10 µg), tetracycline (10 µg) and (25 µg), chloramphenicol (10 µg), ofloxacin (5 µg), nalidixic acid (30 µg), nitrofurantoin (20 ug), amoxicillin (25 µg), cotrimoxazole (25 µg) vancomycin (5 µg) and methicillin (5 µg) (Abtek biological Ltd, Liverpool, UK). The concentrations of antimicrobial sensitivity and interpretation of sizes of zones of inhibition were in accordance to Performance Standards for antimicrobial disk susceptibility tests, CLSI (2004) and WHO (2001) breakpoints. The disk diffusion sensitivity was used to determine resistance for both methicillin and vancomycin. The MIC or E-test which are the gold standard for determining vancomycin susceptibility was not used. The method of Hill et al. (2005) was used in identifying multidrug resistant *S. aureus* isolates, the multi antibiotic resistance index (MAR) as well as their plasmid profile were analyzed.

### Determination of multi antibiotic resistance (MAR) index

MAR index was calculated for bacteria isolates that showed resistance to more than three antibiotics using the method described by Christopher et al. (2013) and Subramani and Vignesh (2012). This was considered as the number of antibiotics to which tested isolates were resistant to, divided by the total number of antibiotics to which the organism was tested against for sensitivity.

### Isolation of plasmid DNA

Plasmid DNA was isolated using the methods described by Birnboim and Doly (1987) with the modifications described by Ombui et al. (2000). All samples were analyzed at the University of Nigeria, Microbiology division. All tubes gel electrophoresis was carried out in tris acetate ETDA buffer containing ethidium bromide for 4 h. A UV transilliminator was used to view the plasmids and photographs taken. Plasmid sizes were estimated from a standard curve drawn of the molecular sizes of the 1.0 Kb distance (Ombui et al., 2000). The strains were grouped based on their molecular weight (MW). Those that had the same MW profile were placed into the same plasmid profile group.

### Plasmid curing

Acridine orange treatment method described by Esinome et al. (2010) was used for curing the resistant plasmids of the bacterial isolates.

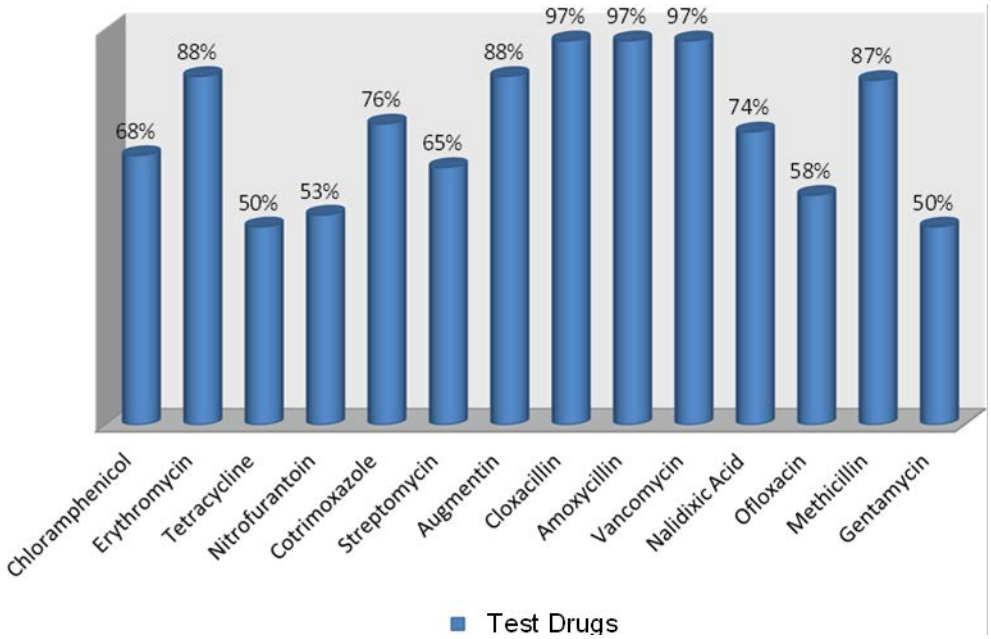


**Figure 1.** Percentage (%) resistance pattern of isolates to commonly used antibiotics.

**Table 1.** Multi-antibiotic resistance (MAR) index analysis for 19 *S. aureus* isolates.

| Isolates serial number | Number of antibiotic to which isolate was resistant to (a) | MAR Index (a/b) |
|---|---|---|
| 1 | 14 | 1.00 |
| 2 | 13 | 0.93 |
| 3 | 13 | 0.93 |
| 4 | 14 | 1.00 |
| 5 | 14 | 1.00 |
| 6 | 13 | 0.93 |
| 7 | 12 | 0.85 |
| 8 | 14 | 1.00 |
| 9 | 14 | 1.00 |
| 10 | 14 | 1.00 |
| 11 | 13 | 0.93 |
| 12 | 13 | 0.93 |
| 13 | 13 | 0.93 |
| 14 | 13 | 0.93 |
| 15 | 13 | 0.93 |
| 16 | 14 | 1.00 |
| 17 | 14 | 1.00 |
| 18 | 13 | 0.93 |
| 19 | 13 | 0.93 |

Total number of antibiotics used 14 (b).

## RESULTS

A total of 34 specimens were collected and *S. aureus* was isolated from all the samples. Of these, 19 (56%) of the isolates exhibited multidrug resistance to 14 commonly used antibiotics. Eight (42%) of the 19 MDR isolated *S. aureus* were resistant to all the antibiotics while 11(57.89%), exhibited multidrug resistance, sensitive to a maximum of three of the test drugs and the results are as presented in Figure 1. The figure shows resistance to β-lactams [augmentin (88%), cloxacillin (97%) and amoxycillin (97%), aminoglycocides (50 and 65% for gentamycin and streptomycin respectively] and resistance to quinolones [nalidixic acid (74%) and ofloxacin (59%). Resistance was also observed to be high for other antibiotics such as methicillin (87%) and vancomycin (97%) at 5 µg by disc diffusion method. However, this method for the determination of vancomycin resistance is not the gold standard as we compared only concentration effects. The results on the multi-antibiotic resistance (MAR) index analysis for the MDR isolates are as presented in Table 1. All the 19 MDR isolates had a very high MAR index as the calculated values were greater than 0.2. Also, of the 19 MDR isolates screened for plasmids, 18 (94.74%) had plasmids and the profiles are shown in Figures 2 and 3. The plasmid profile sizes ranged between 4936 to 12,130 bp. The isolates where categorized into nine different plasmid profile groups based on their molecular weight (MW) as is shown in Table 2. The most commonly encountered size ranges were 5552 and 6557 bp representing 4 (21%) of the isolates each, whereas 8456 bp were found in three isolates representing 15.8%. When isolates were subjected to curing, 8(44%) lost their plasmids and where cured and the results are as presented in Table 3. The table also shows that there

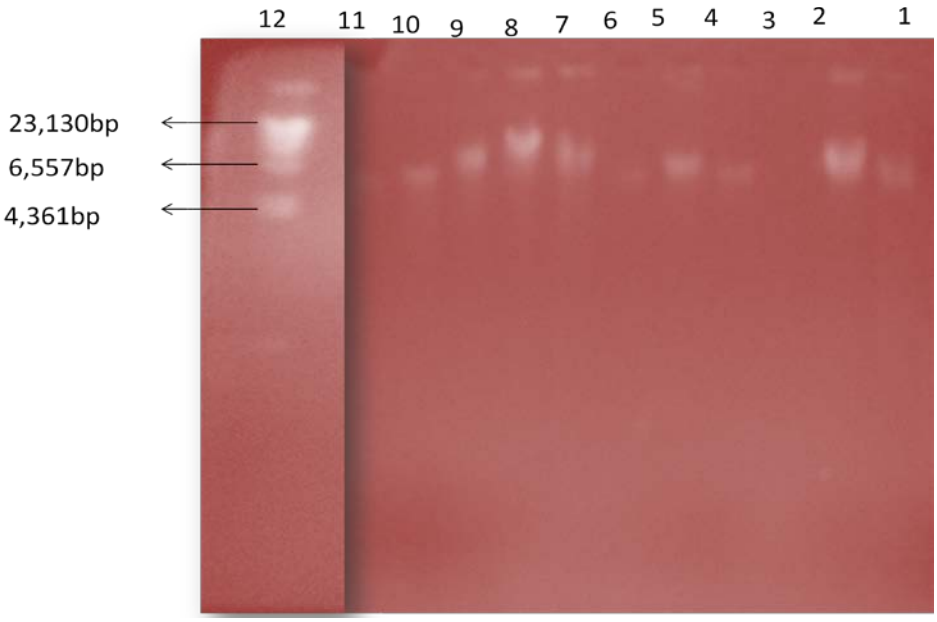


**Figure 2.** Plasmid profile of *S. aureus* isolates from diabetic wounds showing 10 isolates with plasmids. Lane 12 contains the standard reference molecular weight.

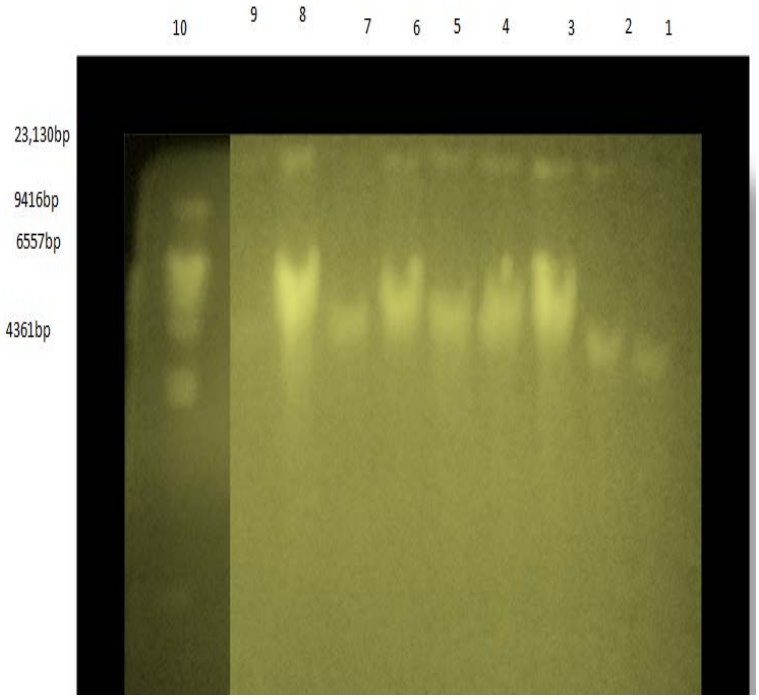


**Figure 3.** Plasmid profile of *S. aureus* isolates from diabetic wounds showing isolate no. 9 without any plasmid. Lane 10 contains the standard reference molecular weight.

was no correlation between the MW of plasmids and the isolates. Some isolates with the same plasmid MW were cured while others in the same group remained uncured.

## DISCUSSION

It is an established fact that bacterial strains, whether

**Table 2.** Plasmid profile of multidrug resistant *S. aureus* isolates grouped into nine different bands.

| Plasmid profile | No. of isolates / (%) | Molecular mass (bp) of isolate |
|---|---|---|
| 0 | 1 (5.3%) | None |
| 1 | 1 (5.3%) | 4936 |
| 2 | 4 (21.1%) | 5552 |
| 3 | 1 (5.3%) | 5861 |
| 4 | 2 (10.5%) | 6148 |
| 5 | 1 (5.3%) | 6253 |
| 6 | 4 (21.1%) | 6557 |
| 7 | 3 (15.8%) | 8456 |
| 8 | 1 (5.3%) | 9416 |
| 9 | 1 (5.3%) | 12,130 |

**Table 3.** *S. aureus* isolates with their plasmid profiles against the result of curing with acridine orange.

| Sample number | Number of antibiotic resistant (before curing) | MW of plasmids (bp) | Exposure to acridine orange |
|---|---|---|---|
| SA.1 | 14 | 5552 | + Growth (not cured) |
| SA.2 | 14 | 6148 | + Growth (not cured) |
| SA.3 | 14 | 5552 | + Growth (not cured) |
| SA.4 | 13 | 6148 | + Growth (not cured) |
| SA.5 | 11 | 5552 | + Growth (not cured) |
| SA.6 | 14 | 6557 | + Growth (not cured) |
| SA.7 | 14 | 6557 | + Growth (not cured) |
| SA.8 | 13 | 12130 | -ve Growth (cured) |
| SA.9 | 13 | 5552 | -ve Growth (cured) |
| SA.10 | 14 | 4936 | -ve Growth (cured) |
| SA.11 | 13 | 5861 | -ve Growth (Cured) |
| SA.12 | 13 | 6253 | + Growth (not cured) |
| SA.13 | 12 | 8456 | + Growth (not cured) |
| SA.14 | 14 | 8456 | -ve Growth (cured) |
| SA.15 | 14 | 6557 | -ve Growth (cured) |
| SA.16 | 10 | 8456 | + Growth (not cured) |
| SA.17 | 13 | 6557 | -ve Growth (cured) |
| SA.18 | 12 | 9416 | -ve Growth (cured) |
| SA.19 | 14 | No plasmid | + Growth (not cured) |

Gram-positive or -negative exhibit resistance to antibiotics worldwide thus creating an unprecedented public health threat. This resistance can sometimes be plasmid base. With the problems of healthcare funding in the developing world, most health care centers still prescribe antibiotics without necessary clinical investigations.

Misuse of antibiotics was reported in South-eastern Nigeria were the present investigation was carried out (Esimone et al., 2007). This behaviour could lead to the spread of MDR bacteria and consequently a change in antibacterial resistance pattern. The finding of plasmids observed in 18 of the 19 MDR isolates in the present study suggests a plasmid based resistance. It would not be unexpected that these patients presenting with wounds at the hospital would probably have tried self medication and have only gone to the health center probably as a last resort. Also, the 19 MDR *S. aureus* diabetic isolates met the criterion of being described as MAR according to the definition of Hill et al. (2005), as they exhibited resistance to the following three classes of antibiotics; β-lactam, aminoglycosides and quinolones. The high level of resistance observed to β-lactam antibiotics in the present study is similar to the findings of

other workers (Chen et al., 2006; Adegoke and Komolafe, 2009). This characteristic high level of *S. aureus* resistance to β-lactam antibiotics is said to be plasmid based (Adegoke and komolafe, 2009). Earlier report stipulated that R-plasmid-mediated antibiotic can spread in an area where there is heavy use of antibiotics (Subramani and Vignesh, 2012; Daini et al., 2006). Nigeria lies in the region of high antibiotic misuse as reported by Esinome et al. (2007).

The exhibition of MDR to most commonly used antibiotics by the isolates in this study could suggest the possibility of MRSA presence in this region. However there is the need for further investigation to confirm this danger.

According to a recent report, MRSA was isolated from human specimens in another area of South-eastern region of Nigeria (Chibuike et al., 2014). It is therefore important to keep a watch on the emergence of MDR superbugs in this region of the world. Plasmid sizes ranged between 4936 to 12130 bp. This finding is not consistent with the work of Esimone et al. (2010) who reported plasmid size range from 11000-18000 bp. Also Daini and Akano (2006) reported plasmid szes of 1.26, 23.13 and 25.12 kb, while plasmid size range of 300-4000 bp was recorded by Chibuike et al. (2014). This therefore indicates that there is variability in plasmid sizes of MDR S. *aureus*.

Also, MW sizes as seen in the present study were not consistent with curing among the 18 isolates with plasmids as each isolates exhibited individual characteristics. Variations in the methods of isolation could have contributed to these differences. Barton et al. (1995) commented on the difficulty in establishing the size limits of plasmids or the real size distribution in any organism because of methods used in isolation of the organisms as well as the characteristics displayed.

The present findings of MDR *S. aureus* while not appearing to be a usual phenomenon in Nigeria, is a worrisome situation. The 94.7% of the MDR isolates in the present study containing plasmids is high when compared with the 41.2% reported by other workers (Diani and Akano, 2009). This further highlights the pressing need to keep a watch on MDR resistant bacteria emerging from this region. Also the result on plasmid curing as seen in the present study means that antibiotic resistance cannot be said to be entirely plasmid mediated.

These findings are similar to those of Chibuike et al. (2014) and contrary to the findings of Daini and Akano (2009) who also reported a complete cure amongst their isolates. No explanation can be given for now, as to why some plasmids with same MW group were cured while others remained uncured.

There is the possibility that resistance for many were plasmid base while the absence of plasmid in one could mean that the some might be chromosomal based. The possibility of the patients self-medicating cannot be over-looked, as well as the over-zealousness to treat every infection with antibiotics by hospital doctors or by the patients.

Antibiotics can easily be bought off the shelves in area of study. Therefore, the high levels of MDR *S. aureus* isolates observed in the present study could be as a result of misuse or undue exposure to antibiotics. Also, for all the isolates in the present investigation, bacterial MAR index was greater than 0.2, implying that the strain of bacteria originated from an environment of high use antibiotics as indicated by Christopher et al. (2013) and Subramani and Vignesh (2012).

## Conclusion

The high level of multi drug resistance *S. aureus* observed in this study could potentially predict a change in resistance pattern of the community. It also suggests that the patients involved in this study are likely from a region of high antibiotic use. This study therefore highlights once again that misuse of antibiotics could possibly lead to a change in microbial resistance characteristics causing treatment failure and increase in the cost of infection control. The fact that the populace from this region can easily buy antibiotics without prescription lays credence to this and therefore such behaviour could lead to a major public health issue that needs to be attended to urgently.

## Conflict of Interest

The author(s) have not declared any conflict of interest.

## REFERENCES

Adegoke AA, Komolafe AO (2009). Multi-drug resistant *Staphylococcus aureus* in clinical cases in Ile-Ife, Southwest Nigeria. Int. J. Med. Med. Sci. 1(3):068-072.

Adeleke OE, Odelola HA, Oluwole FA (2002). Curing of Antibiotic resistance in clinical strains of *Staphylococcus aureus*. Afr. J. Med. Pharm. Sci. 6:19-25.

Akinyemi KO, Alabi SA, Taiwo MA, Omonigbehin EA (1997). Antimicrobial susceptibility pattern and plasmid profiles of pathogenic bacteria isolated from subjects with urinary tract infections in Lagos Nigeria. Nig. Qt. J. Hosp. Med. 7:7-11.

Barton BM, Harding GP, Zuccarelli AJ (1995). A general method for detecting and Sizing Large Plasmids. Anal. Biochem. 226(2): 235-40.

Bhakta M, Arora S, Bal M (2003). Intra-species Transfer of a Chloramphenicol- resistance plasmid of staphylococcal origin. Indian J. Med. Res. 117:146-151.

Birnboim HC, Doly J (1987). A. rapid alkaline extraction procedure Salmonellosis traced to pasteurised milk. J. Amer. Med. Assoc. 258:3269-3274.

Brennan GI, Shore AC, Corcoran S, Tecklenborg S, Coleman DC, O'Connell B (2012). Emergence of hospital- and community-associated panton-valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* genotype ST772-MRSA-V in Ireland and detailed investigation of an ST772-MRSA-V cluster in a neonatal intensive care unit. J. Clin. Microbiol. 50(3):841-847.

Chen KT, Huard RC, Della-Latta P, Lisa S (2006). Prevalence of methicillin-sensitive and methicillin-resistant Staphylococcus pregnant women. Obstet. Gynecol. 108:482-287.

Chibuike I, Reginald AO, Solomon CU (2014). Antibiotic resistant pattern

and plasmid profiles of methicillin resistant *Staphylococcus aureus* isolated from human samples. Br. Microb. Res. J. 4(2):185-194.
Christopher AF, Hora S, Ali Z (2013). Investigation of plasmid profile, antibiotic susceptibility pattern multiple antibiotic resistance index calculation of *Escherichia coli* isolates obtained from different human clinical specimens at tertiary care hospital in Bareilly - India. Ann. Trop. Med. Public Health 6:285-289.
Clinical Laboratory Standards Institute (CLSI) (2004). Performance standards for antimicrobial susceptibility testing; fourteenth informational supplement (M100-S14). 24.
Cowan ST, Steel KJ (2004). In Manual for identification of Medical Bacteria. 3rd Edition.Cambridge University Press. pp. 50-140.
Daini AO, Akano SA (2009). Plasmid Mediated antibiotic resistance in *Staphylococcus aureus* from patients and non patients. Sci. Res. Essay 4(4):346-350.
Daini OA, Ogbolu DO, Ogunledun A (2006). Plasmid determined resistance to Quinolones in Clinical Isolates of Gram-negative Enteric Bacilli. Afr. Med. Sci. 35: 437-441.
De Vriese AS, Vandecasteele SJ (2014). Vancomycin: the tale of the vanquisher and the pyrrhic victory. Perit. Dial. Int. 34(2):154-61.
Dhanoa A, Singh VA, Mansor A, Yusof MY, Lim KT, Thong KL (2012). Acute haematogenous community-acquired methicillin-resistant *Staphylococcus aureus* osteomyelitis in an adult: Case report and review of literature. BMC Infect. Dis. 12:270.
Diep BA, Chambers HF, Graber CJ et al. (2008). Emergence of multi-drug - resistant community - associates Methicillin - resistant *Staphylococcus aureus.* Clone USA 300 in Men who have sex with men. Ann. Intern. Med. 148:1-17.
Dionigi R, Rover F, Dionigi G, Imperatori A et al. (2002). Risk factors in surgery. J. Chemother. 13:6-11.
Esimone CO, Nworu CS, Udeogaranya PO (2007). Utilization of antimicrobial agents with and without prescriptions by outpatients in selected pharmacies in south-eastern Nigeria. Pharm. World Sci. 29(6):655-660.
Esinome CO, Nwosu CS, Harrison GT (2010). Antibiogram and plasmid profile of some Multi - Antibiotics resistant Urinopathogens obtained from local communities of Southeastern Nigeria. Ibnosina J. Med. Biomed. Sci. 2(4):152-159.
Gould IM, David MZ, Esposito S, Garau J, Lina G, Mazzei T, Peters G (2012). New insights into methicillin-resistant *Staphylococcus aureus* (MRSA) pathogenesis, treatment and resistance. Int. J. Antimicrob. Agents 39(2):96-104.
Hill D, Barbara R, Aniko P, et al. (2005). Antibiotic Susceptibilities of *Pseudomonas aeruginosa* Isolates Derived from Patients with Cystic Fibrosis under aerobic anaerobic and biofilm Conditions. J. Clin. Microbiol. 43:5085-5090.
King MD, Humphrey BJ, Wang YF et al. (2006). Emergence of community - acquired methicillin -resistant *Staphylococcus aureus* USA 300 clone as the predominant cause of skin and soft tissue infections. Ann. Intern. Med. 144: 309-317.
Mammina C, Calà C, Bonura C, et al. (2012). EPI-MRSA Working Group: Polyclonal non multi-resistant *Staphylococcus aureus* isolates from clinical cases of infection occurring in Palermo, Italy, during a one-year surveillance period. Ann. Clin. Microbiol. Antimicrob.11: 17.
Mishra SK, Rijal BP, Pokhrel BM (2013). Emerging threat of multidrug resistant bugs -*Acinetobacter calcoaceticus baumannii* complex and Methicillin resistant *Staphylococcus aureus.* BMC Res. Notes 6(98):2-6.
NCCL (2002). National Committee for Clinical Laboratory Standards for antimicrobial disk susceptibility testing. Twelfth information supplement (M100 - S12) Wayne, PA.
Ombui JN, Kimotho AM, Nduhiu JG (2000). Antimicrobial Resistant patterns and Plasmid profiles of *Staphylococcus aureus* isolated from milk and meat. E. Afr. Med. J. 77(9): 463-465
Park B, Liu GY (2012). Targeting the host-pathogen interface for treatment of *Staphylococcus aureus* infection. Semin. Immunopathol. 34(2):299-315.
Subedi S, Brahmadathan KN (2005). Antimicrobial susceptibility patterns of clinical isolates of Staphylococcus aureus in Nepal. Clin. Microbiol. Infect. 11(3): 237-240.
Subramani S, Vignesh A (2012). MAR index study and MDR Character analysis of a few golden Staphylococci isolates. Asian J. Pharm. Life Sci. 2(2): 151-154.
World Health Organization (2001). Improve and expand surveillance. Antibiotic resistance: synthesis of recommendations by expert policy groups. Switzerland: Geneva; 2001. pp. 15-31.

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*Full Length Research Paper*

# An efficient hairy root culture system for *Withania somnifera* (L.) Dunal

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***Withania somnifera*** **is an important aromatic medicinal plant and possesses wide array of pharmacological properties. In the present investigation, an improved version of hairy root culture system was developed by optimizing various transformation parameters such as type of explant, concentration of acetosyringone, Agrobacterium types and co-cultivation period. Between the leaf and cotyledon explants and two *Agrobacterium rhizgenes* strains (R1000 and A4) tested, leaf explants infected with R1000 and cocultured for five days on MS basal half strength medium in the presence of acetosyringone (100 µM) attained a higher frequency (88%) of hairy root induction. By adopting this protocol, we could utilize the hairy root culture for industrial scale production of withanolides.**

**Key words:** Leaf explant, *Agrobacterium rhizogenes*, *Withania somnifera,* co-cultivation period, acetosyringone.

## INTRODUCTION

*Withania somnifera* (L.) Dunal (*Solanaceae*), commonly known as 'ashwagandha' and "Indian ginseng" is a highly valued medicinal plant in Indian Ayurvedic and African traditional systems. Major withanolides like withanolide A and withaferin A present in *W. somnifera* have been demonstrated to possess specific therapeutic action against carcinogenesis, Parkinson's disease and Alzheimer's disease (Mishra et al., 2000). The requirement of dried plant material for withanolides drugs production in India has been estimated as about 9127-tonnes as against the annual production of about 5905-tonnes (Sharada et al., 2008). Moreover, field cultivation is time consuming, laborious and it is not able to meet the current Ashwagandha global market requirement (Sivanandhan et al., 2012b; 2013a): First, the plant-to-plant variation in secondary metabolites yield along with quality and second the long growing period (4-5 years) between planting and harvesting. To improve commercial cultivation of *W. somnifera*, biological advances must be made that should either increase the yield or reduce the time gap and assure quality (Banerjee et al., 1994).

To enhance commercial prospects for production of

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withanolides, an alternative choice could be the use of plant cell/organ cultures. Hairy root cultures offer many advantages over conventional cell culture systems for secondary metabolites production (Georgiev et al., 2007; Sivanandhan et al., 2013b). Hairy roots are adventitious roots derived from cells transformed by the root-inducing plasmid of *A. rhizogenes* and they grow in the absence of phytohormones. The hairy root harbors the T-DNA segment of plasmid Ri within its genome (Tepfer, 1990). Rao and Ravishankar (2002) suggested *A. rhizogenes*-mediated hairy root formation as the valuable tool for the biosynthesis of secondary metabolites, metabolic engineering studies and biotechnological production of root-derived compounds.

*A. rhizogenes*-mediated transformation has been previously reported in *W. somnifera* using various explants (leaf, cotyledon, cotyledonary node, hypocotyls, stem, and root) and various strains (A4, LBA9402, MTCC 2364, MTCC 532, ATCC 15834, LMG 150, A2/83, A20/83, R1000 and R1601) by various authors (Banerjee et al., 1994; Ray et al., 1996; Pawar and Maheswari, 2004; Kumar et al., 2005; Bandyopadhyay et al., 2007; Murthy et al., 2008; Praveen and Murthy, 2011; Sivanandhan et al., 2012a; Sivanandhan et al., 2013b). In the present study, we documented a higher number of hairy roots and transformation frequency upon inclusion of AS and MES buffer in the co-cultivation medium by optimizing different factors in *W. somnifera*. Hence, in the present study, two agropine type *A. rhizogenes* strains (R1000 and A4) have been selected to induce hairy roots in the Kolli Hills genotype of *W. somnifera*.

## MATERIALS AND METHODS

### Plant material

The explants, leaf and cotyledon were prepared as per our earlier report (Sivanandhan et al., 2013b).

### *Agrobacterium rhizogenes*-transformation

A single colony of the wild type *Agrobacterium rhizogenes* strains R1000 and A4 were selected and cultured in LB medium (30 ml; Himedia, Mumbai, India) in darkness at 28°C for 12 h at 180 rpm. The bacterial cells were pelleted by centrifugation followed by washing twice with liquid half strength MS medium (Murashige and Skoog, 1962). The suspension was employed for *A. rhizogenes* infection.

### Standardization of transformation parameters

In order to induce the hairy roots with *A. rhizogenes*, we tested two types of explants, namely leaves and cotyledons. The explants such as leaf and cotyledon were pricked with sterile hypodermic needle (0.63×25 mm; Dispovan, Haryana, India Ltd.) and the explants were immersed in the bacterial suspension culture (OD 600≥1) for 15 min and blotted on sterile tissue paper for 10 min. After dryness, the explants were placed in half strength MS medium supplemented with different concentrations of acetosyringone (AS) (0, 50, 100, 150, and 200 µM; Sigma, St. Louis, USA), 3% (w/v) sucrose (SRL, Mumbai, India), and 0.2% (w/v) phytagel (Sigma, St. Louis, USA), at 23 ± 2°C in dark conditions. These explants were co-cultivated for different days (3, 5 and 7 days). After the co-cultivation period, the explants were washed first with sterilized-distilled water followed by half strength MS medium, which contained 300 mg/l cefotaxime (Alkime Laboratory, Mumbai, India) and transferred to 30 ml half strength MS medium supplemented with 3% (w/v) sucrose, 0.2% (w/v) phytagel, and 300 mg/l cefotaxime. After 12-18 days of culture, the transformed roots were appeared at the wounded sites of the explants. The induced transformed roots of more than 1-2 cm in length were excised from the explants and transferred to 30 ml half strength MS medium supplemented with 3% (w/v) sucrose, 0.2% (w/v) phytagel and 300 mg/l cefotaxime until the residual bacteria have been completely killed.

### Statistical analysis

All the experiments were set up in a completely randomized design and the data were subjected to Duncan's multiple range test using SPSS software version 11.5. The efficiency of hairy root formation was calculated as the percent of respective explants showed hairy root induction out of total number of explants inoculated in a particular treatment.

## RESULTS AND DISCUSSION

### Effect of *Agrobacterium* strains on hairy root induction

The used bacterial strains had significant transformation efficiency on leaf and cotyledon explants in *W. somnifera*. In total of 150 leaf explants infected by R1000, an average of 132 leaf explants produced 28.2 number of hairy roots/leaf explant of at least 3 cm in length after 12 days (Table 1). In total of 150 cotyledon explants infected by R1000, an average of 96 cotyledon explants produced 17.6 number of hairy roots/cotyledon of at least 2 cm in length after 16 days. In the case of A4 strain, 150 leaf explants out of 118.7 explants produced 24.3 hairy roots/explant of at least 2 cm in length after 14 days of culture. In total of 150 cotyledon explants infected by A4, an average of 58.3 cotyledon explants produced 14.5 number of hairy roots/cotyledon of at least 1.5 cm in length after 18 days (Table 1 and Figure 1). Among different bacterial strains tested, R1000 was the most effective strain and stimulated rooting efficiency of 88 and 64% for leaf and cotyledon explants, respectively. A4 strain induced rooting efficiency of 79 and 38% for leaf and cotyledon explants, respectively. Murthy et al. (2008) reported that transformation response for cotyledon and leaf explants of *W. somnifera* showed 3 and 40%, respectively with R1601 strain. In our study, the cotyledon and leaf explants showed 64 and 88% of transformation efficiency, respectively. Banerjee et al. (1994) obtained 0.9 and 0.7% of transformation response in leaf explants using A4 and LBA9402, respectively. Pawar and Maheswari, (2004) showed that 20% of leaf explants produced hairy root induction in *W. somnifera*

**Table 1.** Transformation frequency of hairy roots induced from explants by *Agrobacterium rhizogenes* strains in *W. somnifera* on MS medium supplemented with 100 µM AS at 5 day co-cultivation.

| Bacterial strains-explants | Total number of explants infected | Number of transformed explants | Transformation frequency (%) | After induction of hairy root (days) |
|---|---|---|---|---|
| **R1000** | | | | |
| Leaf | 150 | 132.7±0.24[a] | 88.4[a] | 12 |
| Cotyledon | 150 | 96.3±0.21[c] | 64.2[c] | 16 |
| **A4** | | | | |
| Leaf | 150 | 118.7±0.26[b] | 79.0[b] | 14 |
| Cotyledon | 150 | 58.3±0.22[d] | 38.8[d] | 18 |

Values represent the mean ± standard error of three replicates. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level.

using both MTCC 2364 and MTCC 532. Bandyopadhyay et al. (2007) reported that LBA 9402 and A4 showed equal effect in leaf explants (85%) and observed three morphological phenotypes of transformed roots. Kumar et al. (2005) used various bacterial strains-ATCC 15834, LMG 150, A2/83 and A20/83 for hairy root induction but, they failed to report the effect of bacterial strains in hairy root induction since their study was aimed to test the antioxidant activity in transformed hairy roots. In the present study, the results demonstrated that *W. somnifera* is more susceptible to strain R1000 than strain A4. It has been reported that the virulence of *A. rhizogenes* strain various among plant hosts (Bush and Pueppke, 1991) and that the transformation efficiency of host species can vary between different bacterial strains (Godwin et al., 1991). Similar result was found in *Gentiana macrophyll* hairy root culture. In *G. macrophyll* species, the R1000 strain showed 32% of transformation frequency in matured leaf explant compare to 53% of transformation frequency in stem explants (Tiwari et al., 2007). It was observed from the present study that most hairy roots emerged from the wounded sites of midrib of leaf and cotyledon explants infected by both strains used. It was hypothesized that the cell contains high level of auxin and sucrose are ideal target for hairy root induction (Nilsson and Olsson, 1997). Since the phloem cells, positioned deep in plant organs are supposed to have high sucrose and IAA, they could be the target of *A. rhizogenes*. This might be the possible explanation of observation. Therefore, the *Agrobacterium* strain plays an important role in hairy root induction depending on explants in *W. somnifera.* This study strongly suggests that R1000 was a more effective strain in hairy root induction from 45-day-old leaf explants.

## Effect of acetosyringone on hairy root induction

During induction of transformed roots, every individual hairy root was counted to evaluate the effect of aceto-syringone concentration. For activation of 0-200 µM AS in co-cultivation medium, the transformed hairy roots formed from the wounded regions of leaf and cotyledon explants were observed on 12-18 days after co-cultivation. This indicates that, AS contributes more crucial enhancement in root formation during co-culture of *A. rhizogenes* with the explants (Figure 2). AS enriched medium had a major effect on hairy root induction in leaf and cotyledon explants infected with R1000 strain. Addition of 100 µM AS in co-cultivation medium resulted to two-fold higher number of hairy roots. Among leaf and cotyledon explants infected by R1000 strain, the leaf explants produced 28.2 number of hairy roots/explant in 5 day co-cultivation period when 100 µM AS was added to the co-cultivation medium (Figure 2). Whereas in cotyledon explant, they produced 17.6 number of hairy roots/explant in 5 day co-cultivation period with the addition of 100 µM AS to co-cultivation medium. Similarly, inclusion of AS to co-cultivation medium caused dramatic improvement in the number of responding leaf and cotyledon explants infected with A4 strain. Addition of AS (100 µM) resulted higher number of hairy roots from leaf explants than cotyledon explants infected by A4 strain. The leaf explants produced 24.3 number of hairy roots/explant in 5 day co-cultivation period whereas an average of 14.5 number of hairy roots/explant was produced in 5 day co-cultivation period in cotyledon explants (Figure 2). When addition of AS in the co-cultivation medium increased beyond 100 µM, there was reduction in growth of hairy roots from leaf and cotyledon explants of *W. somnifera*. When compared to the effect of AS in infectivity of R1000 and A4 strains, the R1000 strain showed best results in leaf explants when the co-cultivation medium contained 100 µM AS. None of the authors have reported the effect of acetosyringone in hairy root induction in *W. somnifera* whereas, Bandyopadhyay et al. (2007) added 200 µM acetosyringone in bacterial culture prior to infection. Moreover number of studies put forth the effect of acetosyringone for higher hairy root induction (Kim et al.,

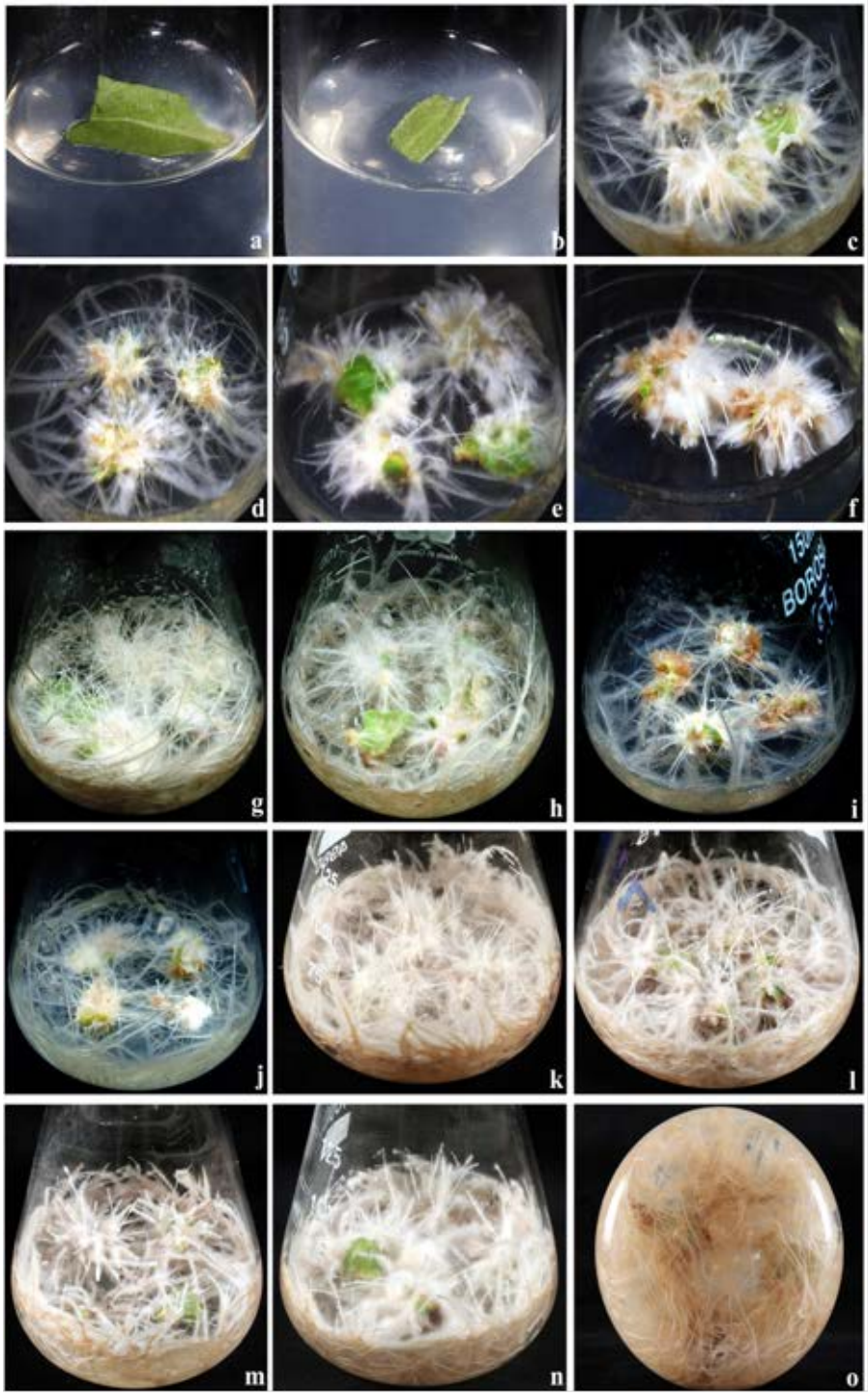


**Figure 1.** High frequency of hairy root induction from leaf and cotyledon explants of *W. somnifera.* **a and b**. Leaf and cotyledon explants placed on co-cultivation medium supplemented with 3 % sucrose containing 100 µM acetosyringone. **c and d.** Hairy root induction from leaf and cotyledon explants infected with R1000 strain after 12 days. e & f Hairy root induction from leaf and cotyledon explants infected with A4 strain after 12 days. g & h Hairy root formation from leaf and cotyledon explants infected with R1000 strain after 16 days. **i and j.** Hairy root formation from leaf and cotyledon explants infected with A4 strain after 16 days. **k and l**. Hairy root formation from leaf and cotyledon explants infected with R1000 strain after 18 days**. m and n**. Hairy root formation from leaf and cotyledon explants infected with A4 strain after 18 days. **o.** Bottom view of hairy root formation from leaf explant infected with R1000 after 12 days.

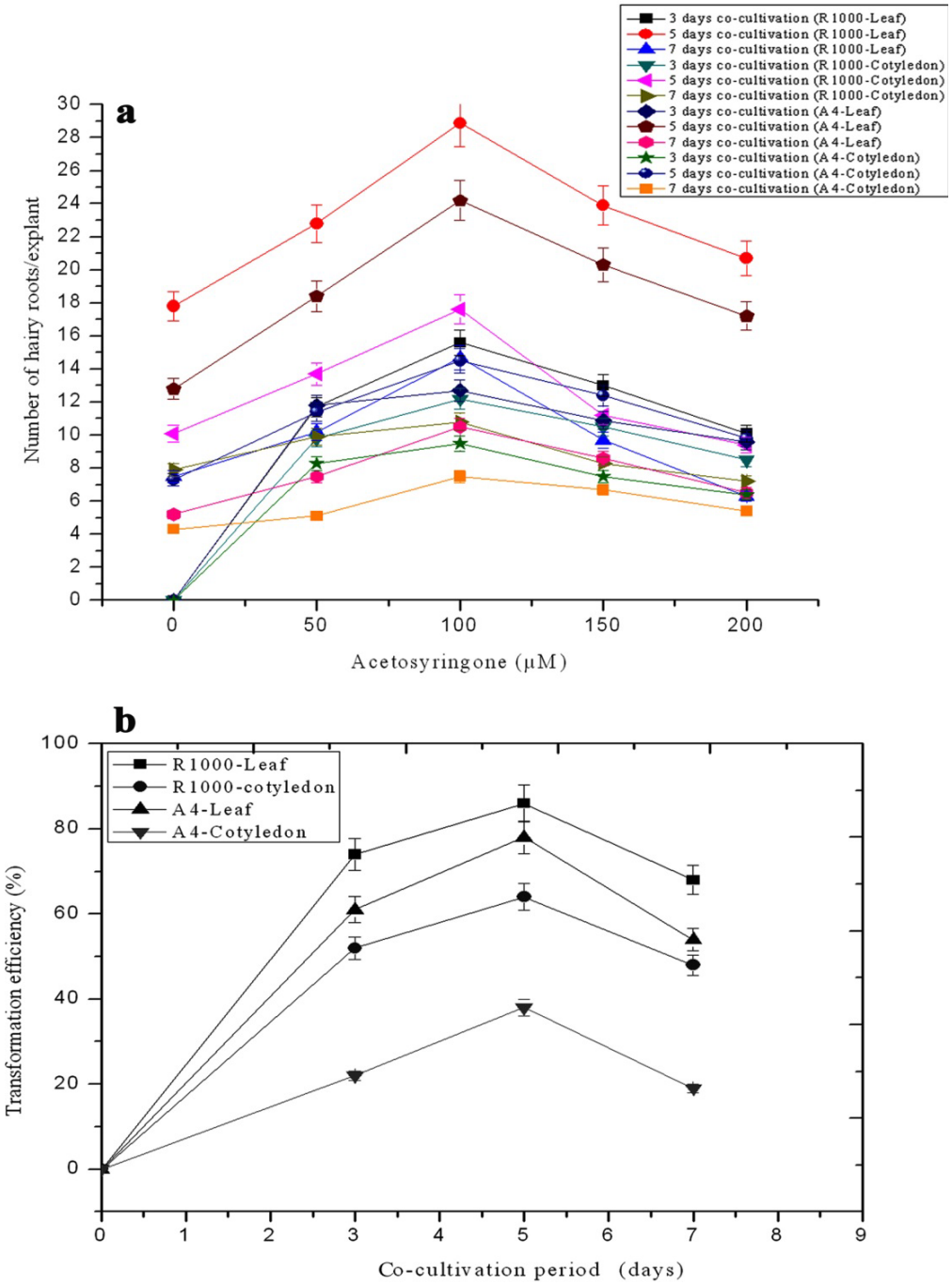


**Figure 2.** Effect of AS and co-cultivation periods on hairy root induction in *W. somnifera*. **a)** AS supplementation in co-cultivation medium in hairy roots induction from both explants of *W. somnifera* infected by R1000 and A4 strains. **b)** Co-cultivation period on transformation efficiency in hairy roots formation infected by both R1000 and A4 in leaf and cotyledon explants of *W. somnifera*. Values represent the mean ±standard error.

2007). Acetosyringone, reported as a virulence inducer to *Agrobacterium*, promotes *Agrobacterium* mediated infection of plants (Stachel et al., 1985). It is widely used as an effective enhancer for *Agrobacterium* mediated plant transformation. A number of plant species, their mediating *Agrobacterium* transformation, and an exogenous supplement of AS have been reported as applied through pre-treating the explants (Sunikumar at al., 1999) or *Agrobacterium* culture (Gelvin and Liu, 1994). Moreover, by inclusion of AS in the culture medium

(Godwin et al., 1991) and combining the pretreatment of explants and *Agrobacterium* culture (Boase et al., 1998) had also been found to enhance the efficiency of *Agrobacterium*-mediated transformation. Thus, we demonstrated that 100 µM acetosyringone along with optimal co-cultivation days are essential for rapid induction of hairy roots from 45-day-old leaf explants of *W. somnifera*.

**Effect of co-cultivation on hairy root induction**

A co-cultivation for 5 days increased the efficiency of the leaf explants with hairy root formation, as it was not observed to obtain a higher number of hairy roots than remaining co-cultivation period. A 5 day co-cultivation period induced 28.2 and 24.3 number of hairy roots/leaf explants infected with R1000 and A4 strains, respectively. In the case of cotyledon explant, the co-culture for 5 days produced 17.6 and 14.5 number of hairy roots/explants infected with R1000 and A4 strains, respectively (Figure 2). Prolonged co-cultivation to 7 days resulted to growth of the bacteria around the both explants which led to explant necrosis. Shorter co-cultivation period may result to an unsuccessful gene transfer from strain to explant. So, the transformation efficiency may differ due to different co-cultivation periods (Figure 2). Of different co-cultivation period tested, 5 day co-cultivation time duration showed dramatic improvement in hairy root induction from the leaf and cotyledon explants, infected with R1000 and A4 strains in *W. somnifera.* A co-cultivation period for 2 and 3 days was found to be suitable for hairy root induction in *W. somnifera*, as reported by Pawar and Maheswari, (2004), Murthy et al. (2008), Banerjee et al. (1994), Kumar et al. (2005) and Bandyopadhyay et al. (2007), but Ray et al. (1996) did not mention the co-cultivation duration, whereas in the present study, 5 day co-cultivation period was found to be superior and abundantly increased the efficiency of hairy root induction (Figure 2). These variations in the requirement for a definite co-cultivation period may originate in the specificity of the plant tissue, the *Agrobacterium* strain or the medium used for bacterial culture and co-cultivation. Co-cultivation is an important phase in transfer of gene from bacteria to plant. But co-cultivation period produced successful transformants on depending upon the plant species and time duration. For R1000 strain, transformation efficiency increased in line with co-cultivation duration and stability on 5th day of culture. During the co-cultivation period, many factors influence the efficiency of the genetic transformation process. After *Agrobacterium* infection, the plant tissues and bacteria are cultivated for a few days. Important events occur during co-cultivation; plant cells and bacteria divide further, and T-DNA is transferred from the bacteria to the plant cells (Kim et al., 2007). The length of the co-cultivation period also influences transformation efficiency (Tao and Li, 2006). These results confirm that the optimization of co-cultivation period is critical for higher hairy root induction in *W. somnifera*.

**Effect of explants on hairy root induction**

Among the various explants tested, only leaf was a greater explant to the infection of both strains. The leaf explant produced higher number of hairy roots by being infected with both the strains R1000 and A4. But the cotyledon explants showed lower number of hairy roots by being infected with both the strains when compared to leaf explants. The leaf explants infected by R1000 and A4 strains produced 28.2 and 24.3 number of hairy roots/explants, respectively and the cotyledon explants infected by R1000 and A4 strains produced 17.6 and 14.5 numbers of hairy roots, respectively (Figure 2). Most of the authors reported that leaf explants only showed the best response for hairy root induction in *W. somnifera*, but Murthy et al. (2008) obtained lower transformation efficiency in cotyledon explants (3%) whereas in the present study, cotyledon explants also produced hairy roots almost equal to leaf explants. Kang et al. (2006) showed the importance of explant choice by producing more hairy roots of *Aralia elata* on the root segment than on the petiole explant. It has been reported in several previous studies that the morphological patterns and hairy root production characteristics of infected tissues differed substantially (Ottani et al., 1990). These differences in hairy root production may depend upon differences between species, plant organs, or sites of infection. The choices of explants for hairy root induction after infection with *A. rhizogenes* constitute the most salient of the integrated factors for a successful hairy root transformation. Plant transformation efficiency differs significantly according to the source of the explant (Alpizar et al., 2006). Therefore, leaf explants possibly influence the rate of hairy root induction than the cotyledon explants by optimizing different factors such as co-cultivation, acetosyringone and bacterial strain in *W. somnifera*.

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**Conflict of Interests**

The author(s) have not declared any conflict of interest.

**REFERENCES**


Alpizar E, Dechamp E, Espeout S, Royer M, Lecouls AC, Nicole M, Bertrand B, Lashermes P, Etienne H (2006). Efficient production of *Agrobacterium rhizogenes*-transformed roots and composite plants for studying gene expression in coffee roots. Plant Cell Rep. 25:959-967.

Bandyopadhyay M, Jha S, Tepfer D (2007). Changes in morphological phenotypes and withanolide composition of Ri-transformed roots of *Withania somnifera*. Plant Cell Rep. 26:599-609.

Banerjee S, Naqvi AA, Mandal S, Ahuja PS (1994). Transformation of *Withania somnifera* (L.) Dunal by *Agrobacterium rhizogenes*: Infectivity and phytochemical studies. Phytother. Res. 8:452-455.

Boase MR, Bradley JM, Borst NK (1998). An improved method for transformation of regal pelargonium (*Pelargonium Xdomesticum* Dubonnet) by *Agrobacterium tumefaciens*. Plant Sci. 139:59-69.

Bush AL, Pueppke SG (1991). Cultivar-strain specificity between *Chrysanthemum morifolium* and *Agrobacterium tumefaciens*. Physiol. Mol. Plant Pathol. 39:309-323.

Gelvin SB, Liu CN (1994). Genetic engineering of *Agrobacterium tumefaciens* strains to improve transformation of recalcitrant plant species. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual. Dordrecht, The Netherlands; Kluwer Academic. pp. 1-13

Georgiev MI, Pavlov AI, Bley T (2007). Hairy root type plant *in vitro* systems as sources of bioactive substances. Appl. Microbiol. Biotechnol. 74:1175-1185.

Godwin I, Gordon T, Ford-Lloyd B, Newbury HJ (1991). The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. Plant Cell Rep. 9: 671-675.

Kang HJ, Anbazhagan VR, You XL, Moon HK, Yi JS, Choi YE (2006). Production of transgenic *Aralia elata* regenerated from *Agrobacterium rhizogenes*-mediated transformed roots. Plant Cell Tissue Org. Cult. 85:187-196.

Kim SJ, Lee JY, Kim YM, Yang SS, Hwang OJ, Hong NJ, Kim KM, Lee HY, Song PS, Kim JI (2007). *Agrobacterium*-mediated high efficiency transformation of creeping bentgrass with herbicide resistance. J. Plant Biol. 50:577-585.

Kumar V, Kotamballi N, Chidambara M, Bhamid S, Sudha CG, Ravishankar GA (2005). Genetically modified hairy roots of *Withania somnifera* Dunal: a potent source of rejuvenating principles. Rejuvenation Res. 8:37-45.

Mishra LC, Singh BB, Dagenais S (2000). Scientific basis for the therapeutic use of *Withania somnifera* (Ashwagandha): a review. Altern. Med. Rev. 5:334-346.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Murthy HN, Dijkstra C, Anthony P, White DA, Davey MR, Power JB, Hahn EJ, Paek KY (2008). Establishment of *Withania somnifera* hairy root cultures for the production of withanolide A. J. Int. Plant Biol. 50:975-981.

Nilsson O, Olsson O (1997). Getting to root: the role of the *Agrobacterium rhizogenes rol* genes in the formation of transformed roots. Physiol. Plant. 100:463-473.

Ottani MP, Schel JHN, Hänisch ten Cate Ch H (1990). Variation in structure and plant regeneration of *Agrobacterium rhizogenes* transformed and control roots of the potato cv. Bintje. Plant Cell Tissue Org. Cult. 20:25-34.

Pawar PK, Maheshwari VL (2004). *Agrobacterium rhizogenes* mediated hairy root induction in two medicinally important members of family Solanaceae. Indian J. Biotechnol. 3:414-417.

Praveen N, Murthy HN (2011). Synthesis of withanolide A depends on carbon source and medium pH in hairy root cultures of *Withania somnifera*. Ind. Crop Prod. 35:241-234.

Rao SR, Ravishankar GA (2002). Plant cell cultures: Chemical factories of secondary metabolites. Biotechnol. Adv. 20:101-153.

Ray S, Ghosh B, Sen S, Jha S (1996). Withanolide production by root cultures of *Withania somnifera* transformed with *Agrobacterium rhizogenes.* Planta Med. 62:571-573.

Sharada M, Ahuja A, Vij SP (2008). Applications of biotechnology in Indian ginseng (ashwagandha): progress and prospects. In: Kumar A, Sopory SK (eds) Recent advances in plant biotechnology and its applications. New Delhi: I. K. International Pvt Ltd. pp. 645-667.

Sivanandhan G, Arun M, Mayavan S, Rajesh M, Jeyaraj M, Kapil Dev G, Manickavasagam M, Selvaraj N, Ganapathi A (2012b). Optimization of elicitation conditions with methyl jasmonate and salicylic acid to improve the productivity of withanolides in the adventitious root culture of *Withania somnifera* (L.) Dunal. Appl. Biochem. Biotechnol. 168: 681-696.

Sivanandhan G, Kapil Dev G, Jeyaraj M, Rajesh M, Arjunan A, Muthuselvam M, Manickavasagam M, Selvaraj N, Ganapathi A (2013b). Increased production of withanolide A, withanone, and withaferin A in hairy root cultures of *Withania somnifera* (L.) Dunal elicited with methyl jasmonate and salicylic acid. Plant Cell Tissue Org. Cult. 114:121-129.

Sivanandhan G, Rajesh M, Arun M, Jeyaraj M, Kapil Dev G, Arjunan A, Manickavasagam M, Muthuselvam M, Selvaraj N, Ganapathi A (2013a). Effect of culture conditions, cytokinins, methyl jasmonate and salicylic acid on the biomass accumulation and production of withanolides in multiple shoot culture of *Withania somnifera* (L.) Dunal using liquid culture. Acta Physiol. Plant. 35: 715-728.

Sivanandhan G, Rajesh M, Arun M, Jeyaraj M, Kapil Dev G, Manickavasagam M, Selvaraj N, Ganapathi A (2012a). Optimization of carbon source for hairy root growth and withaferin A and withanone production in *Withania somnifera*. Nat. Prod. Commun. 7:1271-1272.

Stachel SE, Messens E, Van Montagu M, Zambryski P (1985). Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature 318: 624-629.

Sunikumar G, Vijayachandra K, Veluthambi K (1999). Preincubation of cut tobacco leaf explants promotes *Agrobacterium*-mediated transformation by increasing *vir* gene induction. Plant Sci. 141: 51-58.

Tao J, Li L (2006) .Genetic transformation of *Torenia fournieri* L. mediated by *Agrobacterium rhizogenes*. S. Afr. J. Bot. 72:211-216.

Tepfer D (1990). Genetic transformation using *Agrobacterium rhizogenes*. Physiol. Plant. 79:140-146

Tiwari KK, Trivedi M, Guang ZC, Guo GQ, Zheng GC (2007) .Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes* : growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. Plant Cell Rep. 26:199-210.

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*Full Length Research Paper*

# Effect of starter cultures on microbial and physicochemical parameters of a dry fermented poultry meat sausage

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**The aim of this work was to study the effect of *Staphylococcus xylosus* and *Lactobacillus plantarum* on microbial and physicochemical characteristics of a dry fermented poultry meat sausage. The microbial results show that lactic acid bacteria (LAB) and coagulase negative staphylococci (CNS) represent the two predominant microflora in all stages of ripening. The number of enterobacteria decreased significantly in sausages previously inoculated respectively with *L. plantarum* and with a mixed starter cultures, due to the acidifying and antibacterial activities of lactic acid bacteria. The moisture content of control and inoculated sausages decreased in all stages of ripening which allows a good preservation and consequently, improved their shelf life. Moreover, total free amino acids content increased during ripening of dry fermented sausages. Their concentrations were higher in sausages previously inoculated with starter cultures than in control ones. The main amino acids present in all stages of ripening were aspartic and glutamic acids, arginine, glycine, threonine, alanine, tyrosine, phenylalaline, leucine and isoleucine.**

**Key words:** *Staphylococcus xylosus*, *Lactobacillus plantarum*, ripening, dry fermented sausage.

## INTRODUCTION

The demand for meat products with lower fat contents or healthier fatty acid compositions has increased in recent years due to new guidelines recommending reduced saturated fat intake and consumers' desire to lose weight (Akesowan, 2008; Archer et al., 2004; Colmenero, 2000). Several alternative strategies have been used in the manufacture of these products, such as the substitution of red meat by skinless poultry meat. Skinless poultry

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**Abbreviations: LAB,** Lactic acid bacteria; **CNS,** coagulase negative staphylococci

meat contains more protein, less fat, and less cholesterol than red meat (Hu, 2005; Ressurreccion, 2004). The protein of poultry meat is of excellent nutritional quality, and it contains all of the essential amino acids for human consumption (Varnam and Sutherland, 1995). Furthermore, the manufacture of poultry meat products usually costs less than that of similar beef and pork products (Barbut, 2002). An added benefit is that poultry meat is not restricted by most cultural and religious laws, and it is consumed by both Jews and Muslims (Deumier and Collignan, 2003).

Processed poultry meat products offered a favorable environment for the growth of pathogenic bacteria. Different strategies have been used such as fermentation to guarantee food safety and to standardize product properties. Spontaneous fermentation has been applied for thousands years mainly as method of preservation of excess foodstuff and secondarily as a way to obtain spicy and flavorful foods (Drosinos et al., 2007). In fact, during this stage, the major physical, biochemical and microbiological transformations take place such as: acidification, reduction of nitrates to nitrites, formation of nitrosomyoglobin, solubilization and gelification of myofibrillar and sacroplasmic proteins, proteolytic, lipolytic and oxidative phenomen and dehydratation (Casaburi et al., 2008). Nowadays, the use of selected starter cultures for sausage production is becoming increasingly necessary to guarantee food safety, reduce the fermentation times, extend the shelf life and enhance the organoleptic characteristics of the final product (Parente et al., 2001; Drosinos et al., 2005). Most dry fermented sausages are produced with starter cultures combination of lactic acid bacteria (LAB) and coagulase negative staphylococci (CNS) (Ammor and Mayo, 2007).

Lactic acid bacteria (LAB) play an important role in meat preservation and fermentation processes because they affect both the technological properties and the microbial stability of the final product. In fact, the use of LAB is essential for carbohydrate fermentation and lactic acid generation with subsequent pH drop which contribute to the flavor of the final product through the formation of noticeable acidic and vinegary (acetic acid) tastes. At isoelectric pH, muscle proteins coagulate, lose their water-holding capacity and result in the sliceability, firmness and cohesiveness of the final product. Besides, at this acidic environment ripening is favored as well as color development (Drosinos et al., 2007).

Moreover, LAB inhibits growth of spoilage and pathogenic microorganisms through the production of lactic acid and antimicrobial compounds such as bacteriocins, acetic acid, ethanol, acetoin, carbon dioxide and pyruvic acid (Albano et al., 2009). The species most used as commercial meat LAB starter cultures are *Lactobacillus plantarum, Lactobacillus sakei, Lacobacillus curvatus, Lactobacillus fermentum, Lactobacillus brevis, Pediococcus pentoseaceus* and *Pediococcus acidilactici* (Hammes, 1990; Rovira et al., 1994; Schillinger and Lücke, 1987). Whereas, LAB are mainly responsible for acidification, CNS are associated with color and flavor development (Olesen et al., 2004; Leroy et al., 2006). CNS are Gram-positive, catalase positive cocci with antioxidant activities, preventing the formation of off-flavors and rancidity and their nitrate reductase activity is important for color formation (Ravyts et al., 2010). Their proteolytic and lipolytic activities contribute to the sensory quality of fermented sausages through the generation of flavor-active compounds and their precursors, such as amino acids, free fatty acids, aldehydes, amines, ketones and alcohols (Montel et al., 1996; Leroy et al., 2006). Unfortunately, CNS are generally poorly competitive in a fermented sausage environment, resulting in poor growth and even a decrease in viability during manufacturing (Di Maria et al., 2002; Tjener et al., 2004; Casaburi et al., 2008). The species most used for the production of dry fermented sausages are *Staphylococcus carnosus* and *Staphylococcus xylosus* (Laukova et al., 2010).

In Tunisia, sausages are mainly produced from red meat. The aim of this work is to produce a skinless poultry meat sausage previously inoculated with selected starters of *L. plantarum* and *S. xylosus* and to study the microbial and physicochemical quality during ripening.

## MATERIALS AND METHODS

### Bacterial strains and culture condition

The *S. xylosus* and the *L. plantarum* strains used in this study were both isolated from a Tunisian traditional salted meat “kadid” as previously reported by Essid et al. (2007) and Essid et al. (2009). These strains were selected for having the best attributes for use as starter meat cultures (Essid et al., 2007; Essid et al., 2009). Strains of *S. xylosus* were cultivated in Trypton Soy Broth (Biolife, Italy) at 37°C, whereas strains of *L. plantarum* were cultivated in Man Rogosa and Sharp Broth (MRS broth) (Biolife, Italy) at 30°C. After 24 h of incubation, the bacterial suspensions were centrifuged at 12 000 *g* for 15 min (Universal, 320) and the pellets were washed with sterile distilled water and resuspended in 10 ml of the nutrient broth (Pronadisa, Spain). Cells were harvested by centrifugation (12 000 *g* for 15 min) and resuspended in 5 ml of distilled water. Finally, the number of bacterial cells in each suspension was adjusted to reach the range of 6 log CFU/ml (λ= 600 nm, OD= 0.5 for *L. plantarum*, OD = 0.6 for *S. xylosus*) by using a spectrophotometer (Jenway 6305).

### Preparation of dry fermented sausages

The sausage formulation included 7.5 kg of poultry meat (75%), 2.5 kg of poultry fat (25%), 400 g of salt (4%), 20 g of black pepper (0.2%), 20 g of paprika (0.2%), 100 g glucose (1%) and 1 g of potassium nitrate (0.01%). After chopping and mixing the ingredients, the mixture was divided into four batches (2.5 kg for each batch): batch 1, inoculated with *S. xylosus* alone; batch 2, inoculated with *L. plantarum* alone; batch 3, inoculated with *L. plantarum* and *S. xylosus* and batch 4, control without inoculation. All the samples were inoculated with about $10^6$ viable cells per g of sausages. The mixture of each batch was stuffed into artificial casings, giving approximately 500 g as the final mass of each sausage and then placed in a fermentation chamber (BCR, CF 1 B,

France). The sausages were fermented for 5 days at 24°C and (80%) RH. After five days of processing, the temperature was decreased to 14°C for 23 days. For sampling, three sausages of each batch at 0 day (mix before stuffing) and after 7, 14, 21 and 28 days of ripening were taken for microbiological and physico-chemical analyzes and each analysis was carried out in triplicate.

### Microbiological analysis

Sausage samples (10 g) of each batch were homogenised with 90 ml of sterile peptone water (Biolife, Italy) and decimal dilutions were prepared. Mesophilic lactic acid bacteria (LAB) were enumerated on MRS Agar (Biolife, Italy) after 48 h incubation at 30°C. The number of staphylococci was determined on Mannitol Salt Agar (Biolife, Italy) after incubation at 37°C for 48 h. Yeasts and molds were enumerated on Sabouraud Dextrose Agar (Biokar, France) at 28°C for 4 days.

Total viable counts were determined on Standard Plate Count Agar (PCA) (Biolife, Italy) at 30°C for 48 h. *Enterobacteriaceae* were determined on Violet Red Bile Glucose (VRBG) (Biokar, France) at 37°C for 24 h.

### Physicochemical analysis

#### *pH, moisture and weight loss*

The pH values were measured in homogenates prepared by blending 10 g of sausage (Moulinex DPA141, France) with 50 ml of distilled water for 2 min. Measurements were taken with a pH meter (Microprocessor pH meter BT-500, BoecoGermany). The moisture percentage was calculated by weight loss experimented by the sample (5 g) maintained in an oven (Memmert, UL 60) at 105°C, until constant weight according to the ISO recommended method (ISO, 1973). Weight loss was expressed as a percentage of the initial weight (Liaros et al., 2009).

#### *Color measurement*

Color measurements were carried out using a CR-300 colorimeter (Minolta Chroma Meter CR-300, Japan). Each sausage was cut and the color of the slices was measured three times for each analytical point L*, a* and b* scale coordinates were obtained: L* (lightness), a* (redness) and b* (yellowness). Before each series of measurements, the instrument was calibrated using a white ceramic tile.

### Free amino acids content (FAA)

The content of FAA was determined by reverse phase HPLC. The amino acids were extracted after hydrolysis of meat proteins in the presence of concentrated hydrochloric acid. Thus, 5 g of dry fermented sausages were chopped and added to 4 ml of HCl 37% (6 M). The mixture was homogenized and then placed in an oven at 105°C for 24 h. Hydrolysis was stopped by adding approximately 6 ml of NaOH (6 N). Then, the mixture was filtered through a syringe filter and the filtrate was stored at 4°C until injection. The separation of the protein fraction of sausages was performed using HPLC Agilent L 100 system, on a column C18 (250 mm x 4.6 mm dimensions of the column, 5 µm porosity). This system contains: high pressure pump, an automatic injector FLD, FLD detector and control software and acquisition of data Chemstation. The separation was carried out for 30 min, the flow rate of the mobile phase is 1 ml / min. The excitation was at 340 nm and the emission was at 440 nm. The injection solution is composed of 2.5 µl of the borate, buffer supplemented with 0.5 µl of the sample, 0.5 µl of $H_2O$ and 0.5 µl of *o*-phthaldialdehyde solution. The whole is mixed with 3.5 µl of air. Then, everything was injected. The injection of reference amino acids allowed determining their retention times. To determine the concentration of free amino acids in different samples, straight standards have been established relating the concentration of each reference amino acid to the area of the peak obtained.

### Statistical analysis

Data were statistically analyzed using one-way ANOVA procedure of SPSS® 17.0. Duncan's multiple range test were used to determine any significant difference between mean values and evaluations were based on a significance level of $p < 0.05$.

## RESULTS AND DISCUSSION

### Microbial results

Figure 1 shows the evolution of lactic acid bacteria (LAB), staphylococci, yeasts and molds, *Enterobacteriaceae* and total viable counts during fermentation of starter inoculated and control sausages. Our results show that the addition of selected starter cultures did not affect significantly ($p > 0.05$) the quantitative evolution of different microbial groups with the exception of staphylococci ($p < 0.05$). The growth of microorganisms has been affected significantly ($p < 0.05$) by the time parameter with the exception of staphylococci ($p > 0.05$).

The concentrations of total viable counts in the first day of fermentation were 5.17, 6.53, 6.71 and 6.82 log CFU/g, respectively for control sausages and sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture. During ripening, these numbers increased to reach respectively values of 8.75, 8.92, 8.89 and 9.78 log CFU/g (Figure 1). The numbers of total viable counts remain significantly higher in sausages inoculated compared to those measured on control samples and this at all stages of ripening due to the prior inoculation of sausages by *S. xylosus* and *L. plantarum*. Our results are in agreement with those obtained by Drosinos et al. (2005).

Lactic acid bacteria were the dominant microflora in both control and starter inoculated sausages. This result is in agreement with other studies reporting that LAB are the dominant microflora of dry fermented sausages (Casaburi et al., 2008; Albano et al., 2009; Papamanoli et al., 2003; Bolumar et al., 2006; Ambrosiadis et al., 2004). This predominance is explained by the suitability of LAB to survive in meat environment and their ability to multiply rapidly during ripening (Drosinos et al., 2005). The LAB count in the first day of fermentation was 5.89 log CFU/g for control sausages against 6.14, 7.59 and 7.35 log CFU/g, respectively for sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture. During the first 14th days of ripening, the number of mesophilic LAB increased significantly to reach values of 8.34, 8.27,

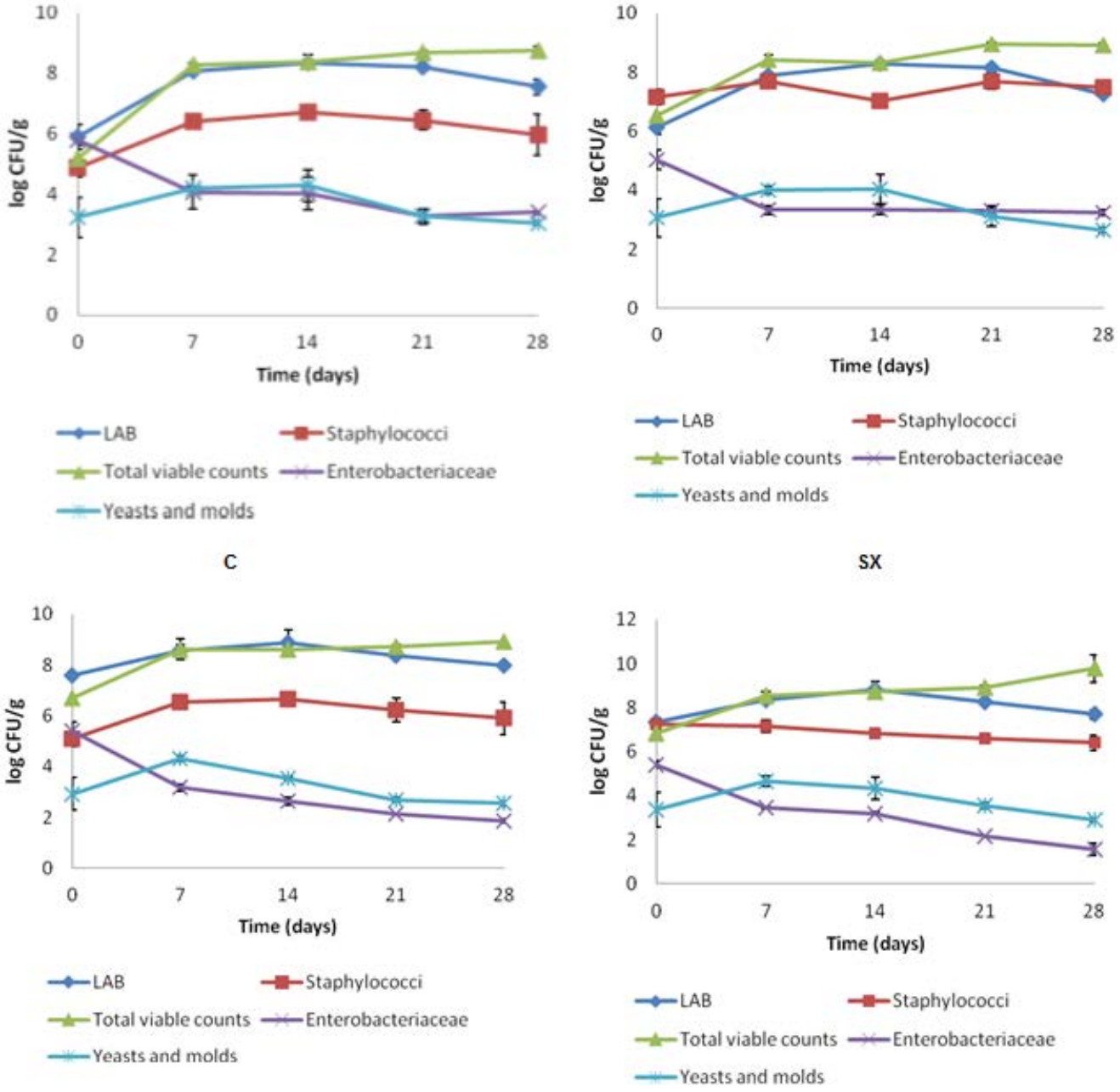


**Figure 1.** Evolution of microbial population during the ripening of control and inoculated dry fermented sausages: **C** (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

8.88 and 8.82 log CFU/g, respectively for control sausages and sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture. Beyond the 14th day, the number of LAB decreased slightly (Figure 1) due to the exhaustion of the sugar (Fernandez-Lopez et al., 2008).

Staphylococci are the dominant microflora next LAB in both control and inoculated sausages. This result is in agreement with many other studies reporting that LAB and staphylococci are the dominant microflora of dry fermented sausages (Casaburi et al., 2008; Rubio et al., 2007). The numbers of staphylococci remain significantly higher in sausages respectively inoculated with *S. xylosus* and mixed starter culture compared to those measured on control samples and samples inoculated with only *L. plantarum* strains. The number of staphylococci increased in control samples and sausages inoculated with *L. plantarum* during the two first weeks of

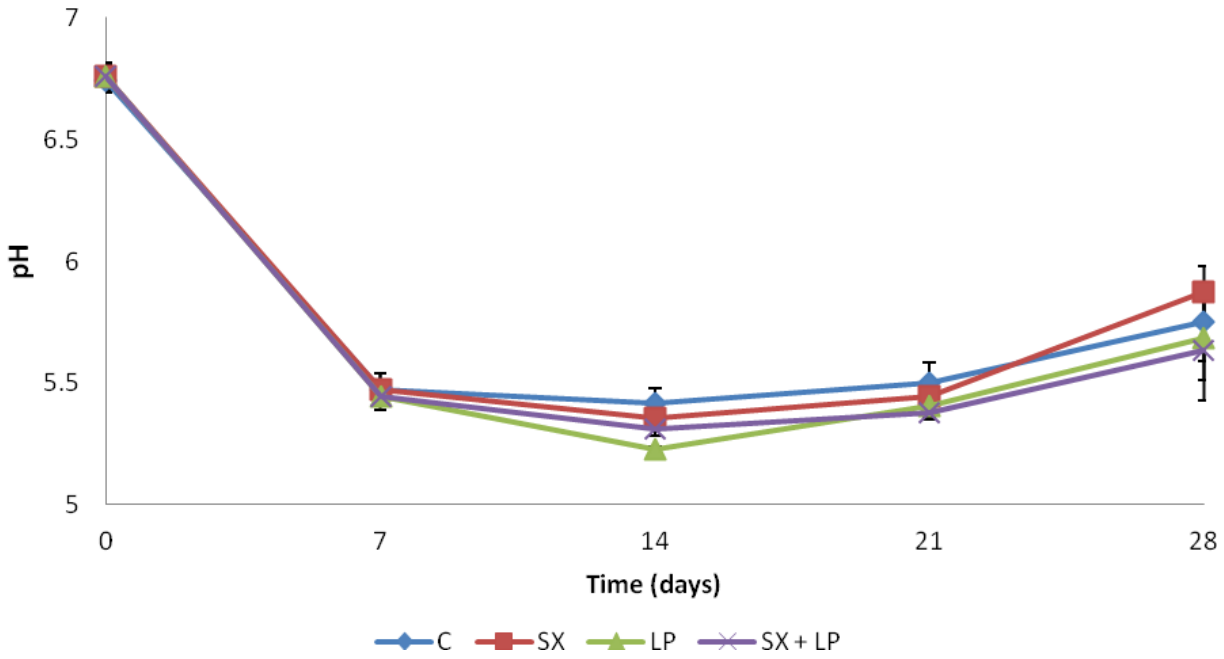


**Figure 2.** Evolution of pH during the ripening of control and inoculated dry fermented sausages: C (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

ripening to reach, respectively at 14th day 6.72 and 6.65 log CFU/g against 7.02 and 6.82 log CFU/g, values measured on samples inoculated respectively with *S. xylosus* and mixed starter culture (Figure 1). The number of staphylococci decreased steadily at the end of ripening and this for all samples of sausages. This inhibition of staphylococci is due to the decrease of pH caused by lactobacilli as reported by other authors (Johansson et al., 1994; Lizaso et al., 1999; Samelis et al., 1998).

The number of *Enterobacteriaceae* decreased during the ripening phase of control and inoculated dry fermented sausages (Figure 1). In fact, during the ripening of sausages inoculated with *L. plantarum* and mixed starter culture, this group showed a strong decrease (99.9%) and reached the level of 1.87 and 1.57 log CFU/g, while, in the control samples and samples inoculated with *S. xylosus,* this group reached the level of 3.39 and 3.24 log CFU/g at the end of ripening ($p < 0.05$). Our results are in agreement with other studies reporting that the sausages inoculated with starter cultures have the lowest number of *Enterobacteriaceae* than in control ones (Casaburi et al., 2008; Papamanoli et al., 2003; Rubio et al., 2007). The reduction of the number of viable cells of *Enterobacteriaceae* is attributed first of all, to the acidification activity of LAB that plays an important role in the inhibition of spoilage and pathogenic microorganisms (Bronomo et al., 2008; Deumier and Collignan, 2003) and secondly, to the antimicrobial compounds excreted by LAB (Ammor and Mayo, 2007; Deumier and Collignan, 2003).

Finally, the number of yeasts and molds increased steadily during the first seven days of maturation and this for all samples of sausages. Then, their concentrations decreased especially in the last two weeks of ripening to reach at 28th day values of 3.03, 2.65, 2.55 and 2.91 log CFU/g, respectively for control samples and sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture (Figure 1). Our results are in agreement with those obtained by Casaburi et al. (2008) who found that the number of molds and yeasts in control and inoculated sausages increased during the first days of ripening and then underwent a reduction to reach at the end of maturation values between $10^2$ and $10^3$ CFU/g.

## pH, moisture and weight loss

The pH values underwent a rapid reduction in control and inoculated sausages from 6.74 ± 0.05 and 6.76 ± 0.05 to reach after 14 days of ripening 5.42 ± 0,06, 5.36 ± 0.05, 5.23 ± 0.01 and 5.31 ± 0.03 respectively for control samples and sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture (Figure 2). This drop in pH is due to the development of lactic acid bacteria that convert sugar added into lactic acid. During the last two weeks of maturation, pH of control and inoculated samples increased gradually. This increase in pH is explained fist of all, by the reduction of the number of LAB due to the exhaustion of the sugar and secondly, to proteolytic activity generated by microorganisms.

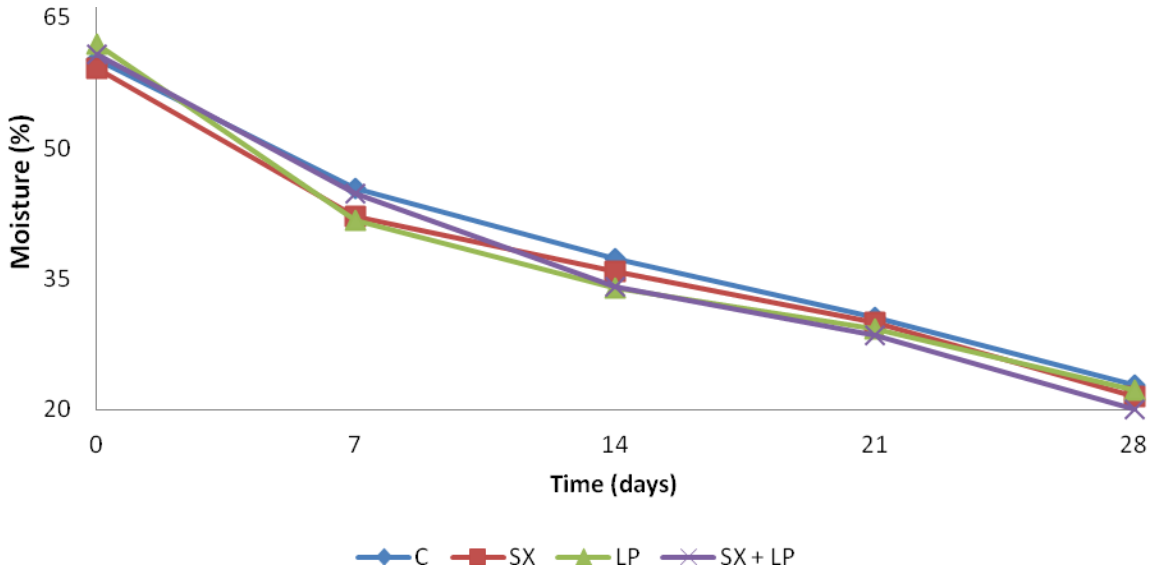


**Figure 3.** Evolution of moisture during the ripening of control and inoculated dry fermented sausages: C (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

Bacterial proteases induce proteolytic degradation, generating peptides, amino acids and amines which have a buffering effect on the organic acids produced by lactic acid bacteria during fermentation (Benito et al., 2007; Ruiz-Moyano et al., 2011).

Our results show that the sausages respectively inoculated with *L. plantarum* and mixture starter culture have a lower pH than in control sausages and sausages inoculate with *S. xylosus*. These results are in agreement with many other studies (Casaburi et al., 2008; Drosinos et al., 2005; Bozkurt et al., 2002) reporting that the pH of dry fermented sausage inoculated with lactic acid bacteria is lower than the pH of control sausage. The moisture content of control and inoculated dry fermented sausages decreased during the ripening phase (Figure 3). This water loss especially during the first two weeks of ripening is due first of all, to the elevated temperature of fermentation (24°C) that accelerates the drying of the product and secondly, to the decrease of pH of sausages to their isoelectric pH which causes protein denaturation and thus a decrease in water retention capacity of myofibrillar proteins (Solignat, 1999).

Our results are in agreement with those of other studies (Jin et al., 2010; Casaburi et al., 2008) reporting that the moisture content of a dry fermented sausages undergoes a decrease during the ripening period. The weight of control and inoculated sausages decreased during the ripening period (Figure 4).

This loss in weight is due to the temperature of fermentation (24°C) that accelerates the dehydration of the product. Our results are in agreement with other studies (Jin et al., 2010; Liaros et al., 2009) reporting that the weight loss of dry fermented sausage increases during the ripening period.

## Color measurement

The color development was significantly affected by the ripening time of sausages ($p < 0.05$) and not by the addition of starters ($p > 0.05$). The quality parameters L*, a* and b* values underwent a decrease through ripening period of different samples studied (Figure 5). Our results are in agreement with those found by Casaburi et al. (2007) and Olivars et al. (2010).

In relation to L* values, a decrease was observed during ripening, since sausage became darker due to weight loss (Olivares et al., 2010). With respect to a* values, a decrease was observed during the first two weeks of maturation, followed by a slight increase. The variation of the parameter color a* during ripening of dry fermented sausages is linked to the formation of a small amount of nitrosomyoglobin pigment (pink-red). Indeed chicken muscle has lower myoglobin content (Mielnik et al., 2002; Yilmaz et al., 2002).

## Free amino acids content

To evaluate the effect of starter of *S. xylosus* and *L. plantarum* on proteolysis, free amino acids were determined through ripening using a chromatographic approach. The concentrations of total free amino acids during ripening are shown in Table 1. Total free amino

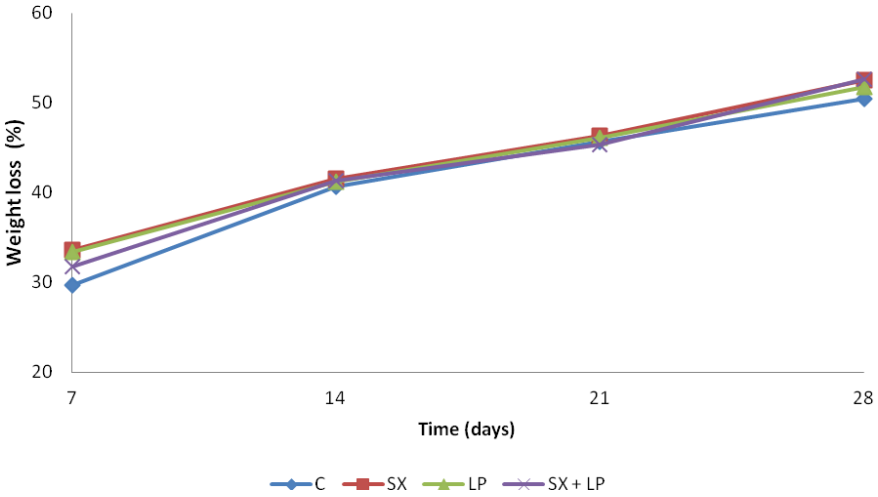


**Figure 4.** Evolution of weight loss during the ripening of control and inoculated dry fermented sausages: C (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

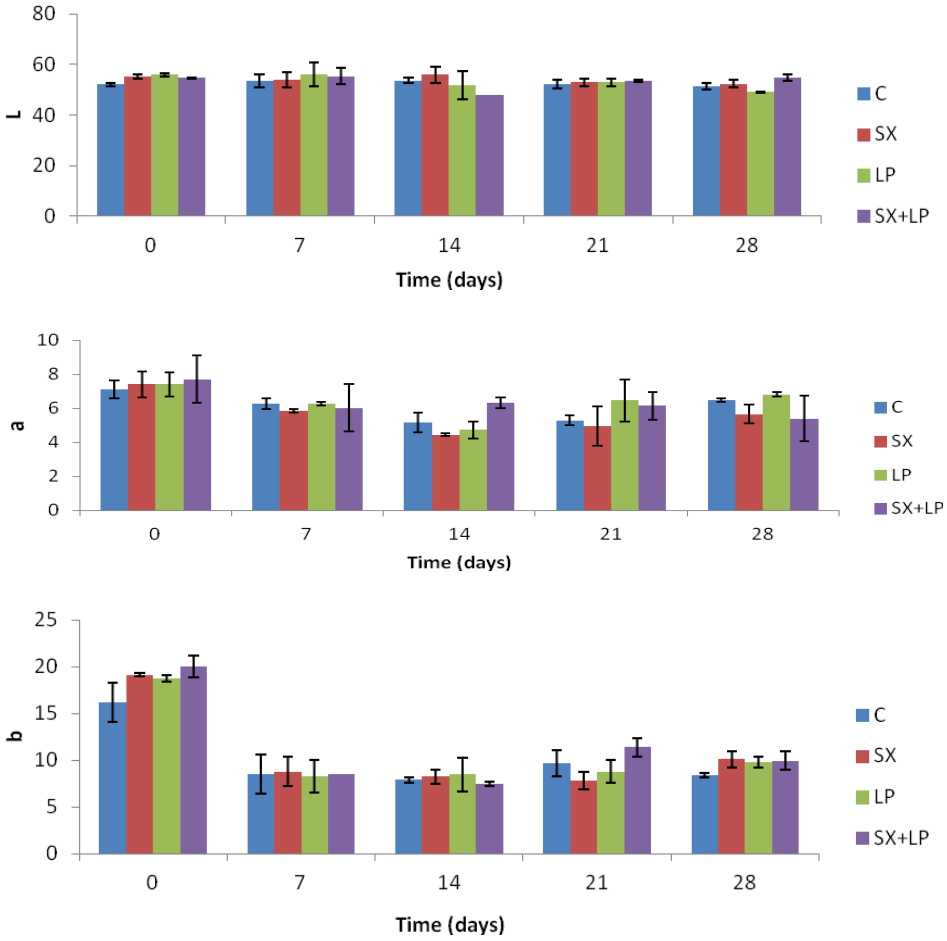


**Figure 5.** Evolution of L*, a*and b* values during the ripening of control and inoculated dry fermented sausages: C (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

**Table 1.** Free amino acids content (mg/kg) during the ripening of control and inoculated dry fermented sausages: C (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

| FAA (mg/kg) | Time (days) | | | | |
|---|---|---|---|---|---|
| | 0 | 28 | | | |
| | C | C | SX | LP | SX+LP |
| Aspartic acid | 109,343 | 75,104 | 106,299 | 132,328 | 99,522 |
| Glutamic acid | 4,787 | 109,082 | 131,731 | 58,884 | 129,499 |
| Serine + histidine+glutamine | 13,792 | 117,549 | 139,175 | 82,373 | 165,283 |
| Arginine + glycine + threonine | 130,150 | 409,206 | 454,428 | 386,513 | 593,840 |
| Alanine | 44,862 | 81,723 | 93,342 | 70,875 | 94,280 |
| Valine + mèthionine + tryptophan | 62,041 | 18,546 | 45,443 | 84,399 | 52,675 |
| Lysine | 19,936 | 3,039 | 0,482 | 19,839 | 8,296 |
| Asparagine | 83,860 | 56,140 | 0,000 | 0,000 | 94,035 |
| Tyrosine | 1238,607 | 1330,547 | 1603,881 | 1512,139 | 1417,612 |
| Phenylalaline | 100,284 | 180,758 | 187,488 | 275,640 | 213,081 |
| Leucine | 123,695 | 313,096 | 312,350 | 233,776 | 314,943 |
| Isoleucine | 128,465 | 251,535 | 258,091 | 420,664 | 278,008 |
| Total | 2059,822 | 2946,325 | 3332,71 | 3277,43 | 3461,074 |

acids content increased significantly during the ripening phase from 2059.82 mg/kg on day 0 to 2946.32, 3332.71, 3277.43 and 3461.07 mg/kg, respectively for control sausages and sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture. Our results are in agreement with those of many other studies (AroAro et al., 2010; Candogan et al., 2009; Casaburi et al., 2008; Lorenzo and Franco, 2012) reporting an increase in total free amino acids content during ripening of sausages. The hydrolysis of meat proteins generates polypeptides that can be further degraded to smaller peptides and free amino acids, this degradation can be produced by endogenous and microbial enzymes as reported by different authors (AroAro et al., 2010; Hughes et al., 2002). The concentrations of total free amino acids during ripening are higher in sausages inoculated than in control samples. This difference in the evolution of total free amino acids are related to the proteolytic activities of endogeneous and microbial enzymes activated by the decrease in pH of the medium ($pH_{op}$ = 4.5 - 5.5) and by the decrease in temperature ($T_{op}$ = 15 - 20°C) (Casaburi et al., 2008).

At the end of the ripening phase, sausages inoculated with *L. plantarum* had the highest concentrations of aspartic acid, valine, methionine, tryptophan, lysine, phenylalanine and isoleucine. While, the highest levels of serine, histidine, glutamine, arginine, glycine, threonine, alanine and leucine were observed in the samples inoculated with the mixed culture. Sausages inoculated with only *S. xylosus* had the highest concentrations of glutamic acid and tyrosine. Moreover, aspartic acid, glutamic acid, glycine, threonine, alanine, phenylalanine, serine, histidine, glutamine, tyrosine, leucine and isoleucine were the dominant amino acids in the four dry fermented sausages. These amino acids play an important role in development of characteristic taste and flavor of the final product (Lorenzo and Franco, 2012; Casaburi et al., 2008).

## Conclusion

The use of starter cultures can improve the safety of the dry fermented sausages by the inhibition of spoilage and pathogenic microorganisms. The number of enterobacteria decreased significantly in sausages previously inoculated respectively with *L. plantarum* and with a mixed starter cultures, due to the acidifying and antibacterial activities of lactic acid bacteria. The moisture content of control and inoculated sausages decreased in all stages of ripening which allows a good preservation and consequently, improved their shelf life.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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## REFERENCES

Akesowan A (2008). Effect of soy protein isolate on quality of light pork sausages containing konjac flour. Afr. J. Biotechnol. 7:4586-4590.

Albano H, Van Reenen CA, Todorov SD, Cruz D, Fraga L, Hogg T, Dicks LMT, Teixeira P (2009). Phenotypic and genetic heterogeneity of lactic acid bacteria isolated from “Alheira”, a traditional fermented sausage produced in Portugal. Meat Sci. 82:389-398.

Ambrosiadis J, Soultos N, Abrahim A, Bloukas JG (2004). Physiochemical, microbiological and sensory attributes for the Characterization of Greek traditional sausages. Meat Sci. 66:279-287.

Ammor MS, Mayo B (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. Meat Sci. 76:138-146.

Archer BJ, Johnson SK, Devereux HM, Baxter AL (2004). Effect of fat replacement by inulin or lupin-kernel fibre on sausage patty acceptability, post-meal perceptions of satiety and food intake in men. British J. Nutri. 91:591-599.

AroAro JM, Nyam-Osor P, Tsuji K, Shimada KI, Fukushima M, Sekikawa M (2010). The effect of starter cultures on proteolytic changes and amino acid content in fermented sausages. Food Chem. 119:279-285.

Barbut S (2002). Poultry products processing. An industry guide. (1st ed.). London: CRC Press.

Benito M, Martin A, Aranda E, Perez-Nevado F, Ruiz-Moyano S, Cordoba MG (2007). Characterization and selection of autochthonous lactic acid bacteria isolated from traditional Iberian dry-fermented sausage Salchichon and chorizo sausages. J. Food Sci. 72:193-201.

Bolumar T, Sanz Y, Flores M, Aristoy MC, Toldra F, Flores J (2006). Sensory improvement of dry-fermented sausages by the addition of cell-free extracts from *Debaryomyces hansenii* and *Lactobacillus sakei*. Meat Sci. 72:457-466.

Bozkurt H, Erkmen O (2002). Effects of starter cultures and additives on the quality of Turkish style sausage (sucuk). Meat Sci. 61:149-156.

Bronomo MG, Ricciardi A, Zotta T, Parente E, Salzano G (2008). Molecular and technologicalogical characterization of lactic acid bacteria from traditional fermented sausages of Basilicata region (Southern Italy). Meat Sci. 80:1238-1248.

Candogan K, Wardlaw FB, Acton JC (2009). Effect of starter culture on proteolytic changes during processing of fermented beef sausages. Food Chem. 116:731-737.

Casaburi A, Di Monaco R, Cavella S, Toldra F, Ercolini D, Villani F (2008). Proteolytic and lipolytic starter cultures and their effect on traditional fermented sausages ripening and sensory traits. Food Microbiol. 25:335-347.

Colmenero FJ (2000). Relevant factors in strategies for fat reduction in meats products. Trends in Food Sci.Technol. 11:56-66.

Deumier F, Collignan A (2003). The effects of sodium lactate and starter cultures on pH, lactic acid bacteria, *Listeria monocytogenes* and *Salmonella spp.* levels in pure chicken dry fermented sausage. Meat Sci. *65*:1165-1174.

Di Maria S, Basso AL, Santoro E, Grazia L, Coppola R (2002). Monitoring of *Staphylococcus xylosus* DSM 20266 added as starter during batch fermentation and ripening of soppressata molisana, a typical Italian sausage. J. Appl. Microbiol. 92:158-164.

Drosinos EH, Mataragas M, Xiraphi N, Moschonas G, Gaitis F, Metaxopoulos J (2005). Characterization of the microbiol flora from a traditional Greek fermented sausage. Meat Sci. 69:307-317.

Drosinos EH, Paramithiostis S, Kolovos G, Tsikouras I, Metaxopoulos I (2007). Phenotypic and technological diversity of lactic acid bacteria and staphylococci isolated from traditionally fermented sausages in southern Greece. Food Microbiol. 24:260-270.

Essid I, Ben Ismail H, Ben Hadj Ahmed S, Ghedamsi R, Mnasser H (2007).Characterization and technological properties of *Staphylococcus xylosus* strains isolated from a Tunisian traditional salted meat. Meat Sci. 77:204-212.

Essid I, Medini M, Hassouna M (2009). Technological and safety properties of *Lactobacillus plantarum* strain isolated from a Tunisan traditional salted meat. Meat Sci. 81:203-208.

Fernandez-Lopez J, Sendra E, Sayas-Barbera E, Navarro C, Perez-Alvarez JA (2008). Physio-chemical and microbiological profiles of « Salchichon » (Spanish dry-fermented sausage) enriched with orange fiber. Meat Sci. 80:410-417.

Hammes WP (1990). Bacterial starter cultures in food production. Food Biotechnol. 4:383-397.

Hu FB (2005). Protein, body weight, and cardiovascular health. American J. Clinic. Nutri. 82:242-247.

Hughes MC, Kerry JP, Arendt EK, Kenneally PM, McSweeney PLH, O’Neill EE (2002). Characterization of proteolysis during the ripening of semi-dry fermented sausages. Meat Sci. 62:205-216.

ISO (1973). Meat and meat products - Determination of moisture. ISO 1442-1973 (E).

Jin G, Zhang J, Yu X, Zhang Y, Lei Y, Wang J (2010). Lipolysis and lipid oxidation in bacon during curing and drying-ripening. Food Chem. 123:465-471.

Johansson G, Berdague´ JL, Larsson M, Tran N, Borch E (1994). Lipolysis, proteolysis and formation of volatile components during ripening of a fermented sausage with *Pediococcus pentosaceus* and *Staphylococcus xylosus* as starter cultures. Meat Sci. 38:203-218.

Laukova A, Simonova M, Strompfova V (2010). *Staphylococcus xylosus* SO3/1M/1/2, bacteriocin-producing meat starter culture or additive. Food Control. 21: 970-973.

Leroy F, Verluyten J, De Vuyst L (2006). Functional meat starter cultures for improved sausage fermentation. Food Microbiol. 106:270-285.

Liaros NG, Katsanidis E, Bloukas JG (2009). Effect of ripening time under vacuum and packaging film permeability on processing and quality characteristics of low fermented sausages. Meat Sci. 83:589-598.

Lizaso G, Chasco J, Beriain J (1999). Microbiological and biochemical changes during ripening of salchichon, a Spanish dry cured sausage. Food Microbiol. 16:219-228.

Lorenzo JM, Franco D (2012). Fat effect on physio-chemical, microbial and textural changes through the manufactured of dry-cured foal sausage lipolysis, proteolysis and sensory properties. Meat Sci. 92:704-714.

Mielnik MB, Aaby K, Rolfsen K, Ellekjær MR, Nilsson A (2002). Quality of comminuted sausages formulated from mechanically deboned poultry meat. Meat Sci. 61:73-84.

Montel MC, Reitz J, Talon R, Berdagué JL, Rousset-Akrim S (1996). Biochemical activities of *Micrococcaceae* and their effectson the aromatic profiles and odours of a dry sausage model. Food Microbiol. 13:489-499.

Olesen PT, Stahnke LH (2004). The influence of environmental parameters on the catabolism of branched-chain amino acids by *Staphylococcus xylosus* and *Staphylococcus carnosus*. Food Microbiol. 21:43-50.

Olivares A, Navarro JL, Salvador A, Flores M (2010). Sensory acceptability of slow fermented sausages based on fat content and ripening time. Meat Sci. 86:251-257.

Papamanoli E, Tzanetakis N, Litopoulou-Tzanetaki E, Kotzekidou P (2003). Cacharacterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. Meat Sci. 65:859-867.

Parente E, Grieco S, Crudele MA (2001). Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in Basilicata (Southern Italy). J. Appl. Microbiol. 90:943-952.

Ravyts F, Steen L, Goemaere O, Paelinck H, De Vuyst L, Leroy F (2010). The application of staphylococci with flavour –generating potential is affected by acidification in fermented dry sausages. Food Microbiol. 27:945-954.

Ressurreccion AVA (2004). Sensory aspects of consumer choices for meat and meat products. Meat Sci. 66:11-20.

Rovira J, Valverde E, Jaime I (1994). Applicación tecnológica de cultivos iniciadores en la elaboración de embutidos crudos curados. Eurocarne. 31:67-70.

Rubio B, Martinez B, Sanchez MJ, Dolores Garcia-Cachan M, Rovira J, Jaime I (2007). Study of the shelf life of a dry fermented sausage “salchichon” made from raw material enriched in monounsaturated fatty acids and stored under modified atmospheres. Meat Sci. 76:128-137.

Ruiz-Moyano S, Martin A, Benito MJ, Hernandez A, Casquete R, Cordoba MG (2011). Application of *Lactobacillus fermentum* HL 57 and *Pediococcusacidilactici* SP979 as potential probiotics in the manufacture of traditional Iberian dry-fermented sausages. Food Microbiol. 28:839-845.

Samelis J, Metaxopoulos J, Vlassi M, Pappa A (1998). Stability and safety of traditional Greek salami—a microbiological ecology study. Int. J. Food Microbiol. 44:69-82.
Schillinger U, Lücke FK (1987). Identification of lactobacilli from meat and meat products. Food Microbiol. 4:199-208.
Solignat G (1999). Drying-Ripening. In: P. Durand (Eds.), Technology of meat and cured products Paris. pp. 278-391.
Tjener K, Stahnke LH, Andersen L, Martinussen J (2004). Growth and production of volatiles by *Staphylococcus carnosus* in dry sausages: influence of inoculation level and ripening time. Meat Sci. 67:447-452.
Varnam AH, Sutherland JP (1995). Meat and meat products. London: Chapman & Hall.
Yilmaz I, Şimşek O, Işikli M (2002). Fatty acid composition and quality characteristics of low-fat cooked sausages made with beef and chicken meat, tomato juice and sunflower oil. Meat Sci. 62:253-258.

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*Full Length Research Paper*

# Towards the development of sweet potato-based couscous for human consumption in Benin

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**Sweet potato processing and consumption patterns are very limited in Benin. The present study aimed to suggest a new utilization of the crop as food. Roots from a white flesh variety were processed into flour and later into couscous. This couscous was steam-cooked following the same procedure as a wheat-based couscous purchased in market. The foods obtained were submitted to panellists' appreciation. Although significant differences were observed between wheat-based couscous and sweet potato-based couscous regarding the colour and the flavour, the mean score obtained by the later couscous showed that the product was acceptable to good sensory quality.**

**Key words:** Sweet potato, couscous, sensory quality.

## INTRODUCTION

Roots and tubers ranked second food crops after cereals, particularly under tropics (Scott et al., 2000; Chandra, 2012). Among these roots and tubers, sweet potato (*Ipomea batatas* [L.] Lam.) has the highest productivity in terms of dry matter, energy and vitamins supply (Bell and Gochenaur, 2006). The Food and Agriculture Organization (FAO) of the United Nations recognized that sweet potato ranked third among root and tubers harvested in the world. Thus, during 2012 year, 108,004,174 tons of sweet potatoes were harvested over the world, with 4,638,664 tons in West Africa (FAO, 2014). Since sweet potato roots are highly perishable, there is a need to process them after harvesting with the purpose of reducing or avoiding wastes.

In Benin, sweet potato contributes to food security for poor families, particularly during lean days. According to FAO (2014) about 70,000 tons were harvested during 2012. Despite its contribution, the crop is still barely considered in the national agricultural development policies.

Sweet potato is processed in various ways over the world. In India, Singh et al. (2004) used sweet potato flour for the development of a pasta product. Processing of dry noodles based on wheat-sweet potato composite flour were experimented by Zhang et al. (2010). In Malaysia, a sweet potato based dessert known as *bubur caca* is consumed (Anonymous, 2011). Candied sweet potato and sweet potato pie are traditional dishes

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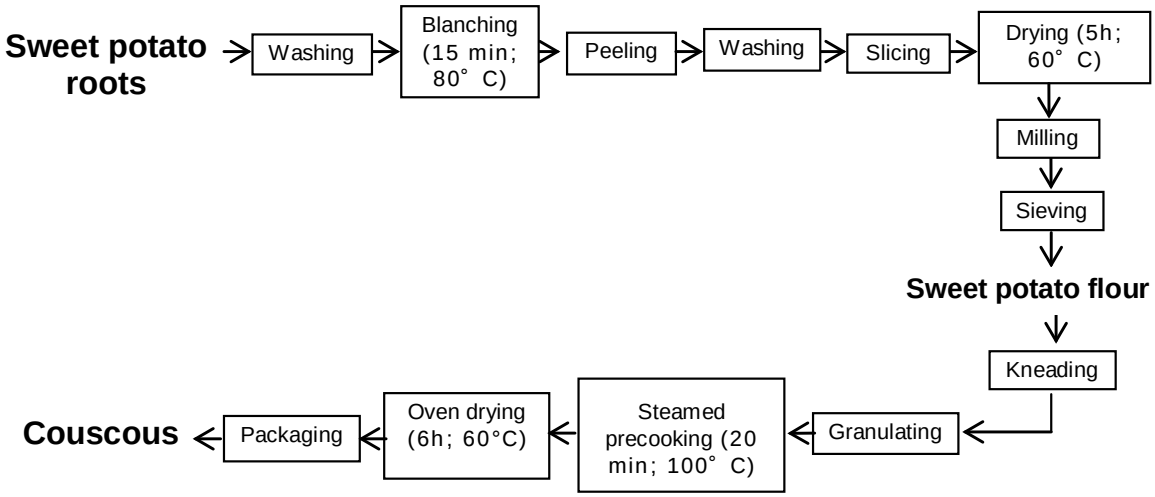


**Figure 1.** Flow diagram of the processing of sweet potato couscous.

consumed in northern America (Anonymous, 2011). Oke and Workneh, (2013) reported the processing of sweet potato into chips or flour for use in bread and cakes making, and its processing into fermented and dried products like fufu in Africa. However, sweet potato processing and consumption patterns are very limited in Benin; that may be the basis of the little interest in this crop. The few processing patterns consist in boiling, roasting or frying of sweet potato. Sometimes the boiled roots are spiced and crushed into a purée. Since the imported wheaten products such as flour, pasta, couscous, etc. are popular on Benin's local markets, and Katan and De Ros (2004) suggested the utilization of indigenous crops such as sweet potato to reduce the importation of wheat and wheaten products in countries where wheat production is disadvantaged,this preliminary study suggested a new utilization of sweet potato through its processing into a couscous for both urban and rural consumers.

## MATERIALS AND METHODS

### Raw material and processing method

The white fleshed variety of sweet potato was purchased from a local market and used during the present study. The processing was carried out in triplicates and followed the procedure summarized in Figure 1. For each repetition, about 4 000 g (Mettler Toledo SB16000, Switzerland) of sweet potato roots were processed.

### Conversion rates calculation

During the processing, products obtained from the raw material to the end product were weighted and finally the conversion rates of sweet potato roots-flour and sweet potato roots-couscous were calculated by doing the ratio of the weight of the product by the initial weight of sweet potato roots.

### Water content determination

Water contents were assessed using the AOAC (2012) methods. Briefly, 5 g sample of fresh sweet potato and its derivatives (flour and couscous) were oven-dried at 105°C for 48 and 24 h, respectively. The evaporated water amount was used to calculate the water contents.

### Sensory evaluation

Couscous from sweet potato was steam-cooked and submitted to 30 untrained consumer's appreciation in comparison with a conventional wheat-based couscous SIPA (PASTACORPS, 180 rue Descartes, France) purchased in a local market. The paired comparison test was used according to the standard 5495: 2005 of the International Organization for Standardization (2010). Comparison was based on colour, taste, texture and flavour of the cooked sweet potato- and wheat-based couscous. Sensory attributes were rated on a 5-point scale going from 1 = unpleasant to 5 = very good.

### Statistical analysis

Data were analysed using SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA). For each sensory attribute the mean score was calculated separately for sweet potato couscous and wheat-based couscous and compared using Student's T-test.

## RESULTS AND DISCUSSION

The processing experiment led to obtaining dry flour and later, dry couscous granules. The technological parameters of the processing are summarized in the Table 1.

It appeared from Table 1 that the conversion rate of

**Table 1.** Technological parameters of the processing of sweet potato roots into couscous.

| Parameter | Sweet potato roots | Sweet potato flour | Sweet potato couscous |
|---|---|---|---|
| Initial weight (g) | 4,000 ± 3.21 | 1,263 ± 88.48 | 2,020 ± 23.12 |
| Conversion rate (%) | - | 31.6 ± 2.2 | 50.5 ± 0.5 |
| Water content (%) | 65.08 ± 0.81 | 5.41 ± 0.67 | 10.84 ± 0.75 |

Results given as averages of triplicate determinations ± S.D.

**Table 2.** Sensory evaluation results analysis using Student T-test.

| Sensory parameter | Paired samples statistics† | | Paired samples test | | | |
|---|---|---|---|---|---|---|
| | Wheat couscous (WC) mean score | Sweet potato couscous (SPC) mean score | 95% confidence interval of the difference | | t value | Significance |
| | | | Lower | Upper | | |
| Colour | 4.30 ± 0.70 | 3.37 ± 1.13 | 0.415 | 1.452 | 3.683 | 0.001** |
| Taste | 4.17 ± 0.79 | 3.40 ± 1.28 | 0.157 | 1.377 | 2.571 | 0.016* |
| Texture | 3.87 ± 1.01 | 3.57 ± 1.17 | -0.265 | 0.865 | 1.087 | 0.286 |
| Flavour | 4.10 ± 0.96 | 3.70 ± 1.24 | -0.248 | 1.048 | 1.263 | 0.216 |

†Results given as averages of 30 determinations ± S.D, **Highly significant, * Significant.

sweet potato roots into flour was about 30% while the conversion rate of sweet potato roots into couscous reached about 50%. This is probably due to the water content of the couscous (10.84%) which was almost 2 times higher than the water content of the flour (5.41%). However, the water content of the processed sweet potato couscous was lower than the maximum threshold of 13.5% requested by the Codex Alimentarius for wheat-based couscous (FAO/WHO, 1995). This ensured the storability of the sweet potato couscous processed in this study.

After steam-cooking, the sweet potato couscous darkened compared to the wheat-based couscous. The difference in colour was evidenced by panellists who detected significant difference (P<5%) between the sweet potato couscous and the wheat couscous regarding their colour and their flavour (Table 2). This phenomenon is similar to the “after cooking darkening” (ACD) of potato resulting from the oxidation of the ferri-chlorogenic acid in the boiled or fried potatoes (Wang-Pruski and Nowak, 2004). The browning of sweet potato products during heat processing was long ago reported by Scott et al. (1944) as the consequence of polyphenol oxidase reaction with tannins when the roots is subjected to tem-peratures not high enough to denature the enzymes. This finding was later confirmed by Ma et al. (1992) who reported that instead of inactivating them, insufficient heat treatment accelerated enzymes activity in sweet potato.

Even though ACD has not any effect on the nutritional value of the food, it was reported as the most widespread undesirable tubers traits (Wang-Pruski and Nowak, 2004). Krishnan et al. (2010) suggested soaking of sweet potato slices in 1.00% acetic acid for 1 h to obtain flour with low browning index. Further investigations will determine methods to be used to avoid the darkening phenomenon during the sweet potato couscous cooking. Both couscous were considered similar (P>5%) as far as the taste and the texture were concerned.

Although significant differences were detected between the processed sweet potato couscous and the wheat-based couscous, more than 50% of panellists considered sweet potato couscous as good to very good for all the sensory attributes tested (Figure 2), showing that the product might be accepted by Benin consumers.

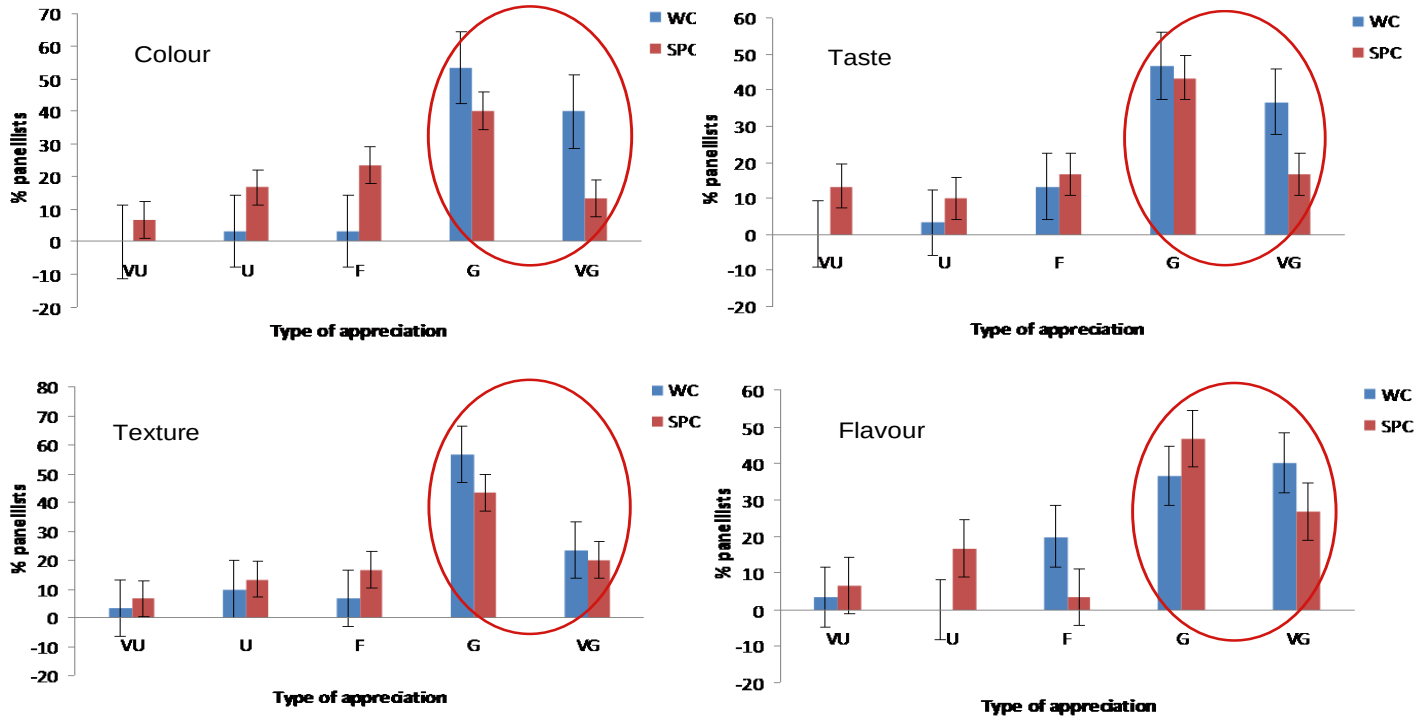


**Figure 2.** Sensory evaluation results: Distribution of panellists according to their appreciation of the couscous per sensory parameter. VU= very unpleasant; U= unpleasant; F= fair; G = good; VG= very good; (■) WC= wheat couscous; (■) SPC = sweet potato couscous.

## Conclusion

The study shows the potential of sweet potato to be used in couscous production. Since Benin is one of the West African countries targeted by the Sweetpotato for Profit and Health Initiative (SPHI) which goal is to "enhance the lives of 10 millions households in 16 Sub-Saharan Africa countries by 2020 through the effective and diversified use of sweet potato" (http://www.sweetpotatoknowledge.org, 2014), this study opens an interesting perspective for sweet potato utilisation, particularly the orange fleshed sweet potato (OFSP) which introduction is in progress in Benin.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

### REFERENCES


Anonymous (2011). http://www.sweetsp.com/. Website accessed September 9th, 2014.

AOAC (2012). Official Methods of Analysis of the Association of Official Analytical Chemists International, 19th edition. Latimer G Jr (Ed.) Rockville, MD 20850, USA.

Bell DR, Gochenaur K (2006). Direct vasoactive and vasoprotective properties of anthocyanin rich extracts. J. Appl. Physiol. 100: 1164-1170.

Chandra SG (2012). Cassava - raw material. http://www.fragrantica.com/news/Cassava-3394.html. Website accessed August 20th, 2014

FAO (2014). Productions en tonnes de la patate douce en 2011. Base des données de FAO. http://faostat.fao.org. Website accessed August 21st, 2014

FAO/WHO (1995). Codex standard for couscous. CODEX STAN 202 , 4p.

http://sweetpotatoknowledge.org/. Sweetpotato Knowledge Portal. Website accessed August 18th, 2014.

International Organization for Standardization (2010). ISO 5495:2005 Sensory analysis Methodology: Paired comparison test. ISO/TC 34/SC 12, Geneva, Switzerland, 21 p.

Katan MB, De Ros NM (2004). Promises and problems of functional foods. Crit. Rev. Food Sci. Nutr. 44:369-377.

Krishnan JG, Padmaja G, Moorthy SN, Suja G, Sajeev MS (2010). Effect of pre-soaking treatments on the nutritional profile and browning index of sweet potato and yam flours. Innov. Food Sci. Emerg. Technol. 11:387-393.

Ma S, Silva JL, Hearnsberker JO, Garner JO Jr (1992). Prevention of enzymatic darkening in frozen sweet potatoes (*Ipomoea batatas* (L.) Lam.) by water blanching: Relationship among darkening, phenols, and polyphenol oxidase activity. J. Agric. Food Chem. 40: 864-867.

Oke MO, Workneh TS (2013). A review on sweet potato postharvest processing and preservation technology. Afr. J. Agric. Res. 8:4990-5003.

Scott G, Best R, Rosegrant MW, Bokanga M (2000). Roots and Tubers in the Global Food System - A vision statement to the year 2020. Peru, Lima, International Potato Centre. pp. 111-117.

Scott LE, Appleman CO, Wilson M (1944). The discoloration of sweet potatoes during preparation for processing and the oxidase in the root. Univ. Md. Agric. Exp. Stn. Bull. A 33:11-26.

Singh S, Singh RC, Singh BA, Chandra SD (2004). Sweet potato-based pasta product: optimization of ingredient levels using response surface methodology. Int. J. Food Sci. Technol. 39: 191-200.

Wang-Pruski G, Nowak J (2004). Potato after cooking darkening. Amer. J. Potato Res. 81:7-16.

Zhang W, Sun C, He F, Tian J (2010). Textural characteristics and sensory evaluation of cooked dry chinese noodles based on wheat-sweet potato composite flour. Int. J. Food Prop. 13: 294-307.

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*Full Length Research Paper*

# Pathogenicity and proteome production of *Isaria fumosorosea* (=*Paecilomyces Fumosoroseus*) (WISE) isolates against lemon butterfly, *Papilio demoleus* (Papilionidae: lepidoptera)

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**The pathogenic potential and catalytic triad conserved amino acids of the isolates *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) (*Ifr$_1$* and *Ifr$_2$*) in response to *Papilio demoleus* was analysed. The isolates showed its potential in killing *P. demoleus* causing mortality of 72.23 and 61.90% at the end of 8 days with $10^8$ spores $ml^{-1}$ concentrations. The enzyme assays (higher proteolytic and chitinolytic activity) also showed that the *Ifr$_2$* was more efficient than *Ifr$_1$*. The predictions of catalytic triads (serine, histidine and asparagine) were also visualized in the peak level obtained in infra-red (IR) and $H_1$ nuclear magnetic resonance (NMR) spectra. With this information it was suggested that, partial characterization of catalytic domain was predicted in the fungal isolates *Ifr.***

**Key words:** Entomopathogenic fungi, *Isaria fumosorosea, Papilio demoleus,* biological control.

## INTRODUCTION

The insect pest management programs heavily rely on the use of synthetic chemical based insecticides or pesticides and herbicides, which is a multibillion dollar industry. The main driving force behind chemical insecticides is the fast speed of kill, high efficacy and political influence of the companies involved in this business. Microbial control is another approach for biological means of plant protection. The use of pathogens in biological control can be integrated with other natural enemies and the immediate effect of a microbial control agent can protect the crops, when parasitoids and predators are unable to maintain the pest population below the economic threshold level.

Fungi are particularly important for controlling sap

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sucking insects (white flies) for which there is no alternative available in the present biocontrol agents. Among them, *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) strains occur in soils and insects world-wide. Their efficacy against *Bemisia argentifolii* and *Trialeurodes vaporariorum* has been described by Wraight et al. (1998) and Fang et al. (1985). Nevertheless, different strains of the same species do not have equal potentials for the control of the same arthropod species (Altre et al., 1999; Vey et al., 1982).

Pathogenicity of an antagonist towards an insect species is related to the ability of the fungus to germinate on the insects' cuticle and to penetrate it, to its production of secondary metabolites and to the defense mechanisms of the host to prevent fungal infection and growth (Kaijiang and Roberts, 1986; Rath et al., 1996; Clarkson et al., 1998; St. Leger et al., 1989). The synthesis of extracellular enzymes is crucial for the infection process of this fungus. Successful infection primarily relies on the synthesis of molecular scissors such as extracellular proteases, chitinases and esterases (Clarkson and Charnley, 1996). The insect infection relies on protease(s) action because 75% of the cuticle is made up of proteins. Furthermore, chitinase(s) help degradation of *N*-acetyl-D-glucosamine moieties present in the cuticle (Charnley, 1997).

Due to the significance of proteases in breaching the insect cuticle, they have received more attention from researcher's worldwide. During more than three decades of research on Entomo-Pathogenic Fungi (EPF), several investigators have established two proteases, namely subtilisins and trypsins, as important virulence factors (St. Leger et al., 1986, 1988). Although the overall fold of various serine proteases may differ, they all follow the same mechanism of action through an identical stereochemistry of the catalytic triad and oxyanion hole. In this mechanism, the serine functions as the primary nucleophile and the histidine plays a dual role as the proton donor and acceptor at different steps in the reaction. The role of asparagine is believed to bring the histidine into the correct orientation to facilitate the nucleophilic attack by the serine. The role of the oxyanion hole is to stabilize the developing negative charge on the oxygen atom of the substrate during the formation of the tetrahedral intermediate (Russell and Fersht, 1987; Dodson and Wlodawer, 1998; Birktoft and Blow, 1972).

In order to increase their utilization, research needs to concentrate on: (a) pathogen virulence and speed of kill, (b) pathogen performance under challenging environmental conditions (cool weather, dry conditions etc.), (c) efficiency in the production process, (d) formulations that enable ease of application, increased environmental persistence and longer shelf-life, (e) integration into managed ecosystems and interaction with the environment and other integrated pest management (IPM) components (Lacey et al., 2001). The three proteases PR1 (*Metarhizium anisopliae*), VCP1 (*Verticillium chladosporium*) and Ver112 (*Verticillium licanii*) have shown that they have a high degree of sequence similarity with each other and belong to the proteinase K family of subtilisin-like serine proteases (subtilases) which is a large family of endopeptidases found only in fungi and Gram-negative bacteria (Siezen and Leunissen, 1997). These enzymes show conservation of the Asp- His-Ser catalytic triad and catalytic domain.

However, the three-dimensional (3D) structures have not been resolved for any of the cuticle-degrading proteases so far by either X-ray crystallography or nuclear magnetic resonance (NMR) techniques. The lacuna of cuticle degrading protease, in the present study reveals that the enzymatic catalytic domain is analyzed through IR and NMR, which provide concrete idea of a particular domain involved in cuticle degradation.

## MATERIALS AND METHODS

### Preparation of the test insects and bioassay

The test lepidopteran insect *Papilio demoleus* (L.) (Papilionidae: Lepidoptera) was maintained on citrus fresh leaves at 27±2°C, 70±5% relative humidity (RH) and 14 h photoperiod under laboratory conditions. Citrus leaves were washed with diluted potassium permanganate solution (0.001%) followed by distilled water to prevent microbial contamination.

Leaves were kept in shade at room temperature until the distilled water evaporates. All the glassware used in the experiments were washed thoroughly in detergent, treated with 2% formalin and then dried in an oven at 70°C for 4 h to check microbial contaminations. The 2nd instar larvae of *P. demoleus* was collected from citrus field near Madurai, Tamil Nadu, India and brought into the laboratory, reared in a wooden cage (60 X 60 cm) providing adequate citrus leaves as a stock culture. The 3rd instar larvae were from stock in this study.

### Isolation protocol of *I. fumosorosea*

Isolation protocol of *Ifr* isolates followed the method of Haraprasad et al. (2001). *Ifr* was isolated from the soil in different locations of Madurai and Theni district, Tamil Nadu. One gram of soil was diluted with 100 ml of distilled water and was serially diluted. From each dilution, 100 µl was placed on PDA medium and it was fortified with streptomycin (10 mg/100 ml). It was allowed to grow for 7 days at 27±2°C (Haraprasad et al*.,* 2001) in the respective media. After 7 days of incubation the fungal colony was identified. The identified fungal colony was sub-cultured in Saboraud Dextrose Agar (SDA) (Hi-Media). The sterilized medium was transferred into sterile Petri dishes (Borosil®) and test tubes (Borosil®) that were then inoculated with conidia by streaking. The isolated fungus *Ifr* was used for the pathogenicity and enzyme studies against *P. demoleus.*

### Efficiency of *I. fumosorosea towards P. demoleus*

The isolates of *I. fumosorosea* were used to determine the pathogenicity of *P. demoleus*. Pure culture of the test fungal species, *I. fumosorosea* isolates was grown on SDA at 27±2°C until a dense sporulating mat was produced (14 days). The conidial suspension of $10^8$ conidia per milliliter was prepared by counting the

**Table 1.** Cumulative mortality (%) of the third instar larvae of *Papilio demoleus* inoculated with various conidia concentrations of the *Irf*$_1$ isolate of *Isaria fumosorosea* .

| Conidial concentration (conidia ml$^{-1}$) | Period (days) after treatment | | | |
|---|---|---|---|---|
| | 2 | 4 | 6 | 8 |
| 1×10$^5$ | 0.00 (1.16)$^{cd}$ | 5.56 (13.63)$^{d}$ | 5.56 (13.63)$^{d}$ | 11.11 (19.47)$^{d}$ |
| 1×10$^6$ | 5.56 (13.63$^{bd}$ | 5.56 (13.63)$^{cd}$ | 11.11 (19.47)$^{cd}$ | 11.11 (19.47)$^{cd}$ |
| 1×10$^7$ | 5.56 (13.63)$^{bd}$ | 33.33 (35.26)$^{b}$ | 50.50 (45.00)$^{b}$ | 55.56 (48.19)$^{b}$ |
| 1×10$^8$ | 27.78 (31.80)$^{a}$ | 38.89 (38.58)$^{a}$ | 55.36 (48.19)$^{a}$ | 72.23 (58.20)$^{a}$ |
| Control | 0.00 (2.86)$^{c}$ | 0.33 (3.29)$^{c}$ | 0.67 (4.69)$^{e}$ | 4.67 (12.48)$^{d}$ |

Each value is mean of three replicates. Values in parenthesis are arc sine transformed values; a - d represents the levels of treatments: 'a' = best treatment and 'd'= pooor treatment.

spores in improved Neubauer counting chamber (Superior Marienfeld, Germany). The conidial suspension per milliliter ($10^5$ - $10^8$ conidia per ml) was prepared for the experimental studies.

Bioassays with different *Ifr* fungal isolates were carried out by dipping 15 third instar larvae of *P. demoleus* in conidial suspensions plus 0.02% Tween 20 at each concentration for 30 s. After 30 s, the larvae was transferred to sterile filter paper and then placed in individual sterilized containers having single citrus leaf previously surface sterilized and was cleaned with sterilized paper towels to eliminate excess water. The bioassay setup was conducted in room at 27±2 °C at 70±5% RH. Each bioassay per concentration was performed in triplicates. A group of larvae (10 in each replicate) was maintained as control treatment; only distilled water plus 0.02% Tween 20. The larvae of *P. demoleus* were observed 2, 4, 6 and 8 days after inoculation with each conidial suspension. The dead larvae were placed in a controlled growth chamber to stimulate the development of fungal mycelia and confirm that the death was by infection of the *Ifr* isolates.

#### Fungal hydrolytic enzymes quantification and separation

The fungal hydrolytic enzyme activities such as α-amylase, proteolytic and chitinolytic activites were determined using Bernfeld (1956), St. Leger et al. (1987) and Ulhoa and Peberdy (1992) methods accordingly.

#### Prediction of catalytic triad of cuticle degrading protease

Ammonium sulphate precipitated culture supernatants were centrifuged at 5000 rpm for 10 min using refrigerated centrifuge. The precipitate was dissolved in the solvent (Butanol: Glacial acetic acid: Water in the ratio of 4:1:5) and then amino acid separations was carried out using Thin Layer Chromatography (TLC). The collected colored fractions were then used for the analysis in the Fourier Transform Infra-Red (FTIR, NEXUS-672 model) and the spectrum was taken in the mid Infra-Red (IR) region of 400-4000 cm$^1$. Nuclear Magnetic Resonance (NMR, Bruker (300 MHz) spectroscopy was also used to to predict the catalytic triad amino acids (asparagine, histidine and serine) of cuticle degrading protease of *I. fumosorosea* secretome. The samples were dissolved by using deuterated chloroform ($CDCL_3$) as solvent.

#### Statistical analysis

Analysis of variance and Duncan's multiple range test (DMRT) was performed to determine the best treatment using SPSS 10 and AGRESS softwares.

## RESULTS

### The efficacy of *I. fumosorosea* on *P. demoleus*

The present study reveals the efficiency of *I. fumosorosea* isolates against *P. demoleus* at various spore concentration of $10^5$ to $10^8$ at different days ($2^{nd}$, $4^{th}$, $6^{th}$ and $8^{th}$ days) of post treatment (Tables 1 and 2). It was observed that, 72.23% (75%) mortality was found in *Ifr*$_1$ (Azhagar kovil) isolate on 8 days at $10^8$ spore/ml whereas only 61.90% mortality was verified by *Ifr*$_2$ isolate. The control treatment unveiled the least mortality of *P. demoleus* by the tested isolates. The mortality due to *Ifr*$_2$ isolate at the initial spore concentration ($10^5$) was at the average of 4.76% only. The mean mortality of the *Ifr*$_2$ isolate ranged from 3.57 to 36.90%. Furthermore, by the 6 days at $10^8$ concentrations only the *Ifr*$_1$ isolate promoted 50% mortality towards *P. demoleus.*

Enzymatic role of *I. fumosorosea* in the pathogenesis of *P. demoleus* was analysed quantitatively by different hydrolytic enzyme assays such as α-amylase activity, proteolytic activity and chitinolytic activity and are represented in Figures 1 and 2. Supernatant obtained from minimal medium in the presence and absence of *P. demoleus* exoskeleton were double filtered after 3 days of incubation and comparatively higher protein secretion was found in the Minimal Medium + Cuticle (MMC) by the *Ifr*$_1$ than MM and *Ifr*$_2$. This may also have helped the *Ifr*$_1$ isolate to contribute higher percent mortality than *Ifr*$_2$.

### Structural elucidation of catalytic triad

Prediction of catalytic triad conserved amino acids of cuticle degrading protease such as serine, histidine and asparagine in the fungal secretome was carried out using FTIR and $^1$H NMR with their basic structures (Figures 3 and 4).

## DISCUSSION

Naturally occurring entomopathogens play an important role in our ecosystem. Invertebrates, viruses, bacteria and fungi can be found as regulatory factors in insect populations. Hence, many species are used as biological control agents of insect pests in row and glasshouse crops, orchards, turf, stored products and forestry and for abatement of vector insects of veterinary and medical

**Table 2.** Cumulative mortality (%) of the third instar larvae of *Papilio demoleus* inoculated with various conidia concentrations of the *Irf*$_2$ isolate of *Isaria fumosorosea*

| Conidial concentration (conidia ml$^{-1}$) | Period (days) after treatment | | | |
|---|---|---|---|---|
| | 2 | 4 | 6 | 8 |
| 1×10$^5$ | 0.00 (1.08) $^c$ | 4.76 (12.60)$^d$ | 4.76 (12.60)$^d$ | 4.76 (12.60)$^d$ |
| 1×10$^6$ | 4.76 (12.60)$^{bd}$ | 14.26 (22.20)$^c$ | 19.04 (25.87)$^c$ | 23.80 (29.20)$^c$ |
| 1×10$^7$ | 4.76 (12.60)$^{bd}$ | 19.04 (25.87)$^{bc}$ | 23.80 (29.20)$^{bc}$ | 48.61 (43.63)$^b$ |
| 1×10$^8$ | 19.04 (25.87)$^a$ | 28.57 (32.31)$^a$ | 38.09 (38.11)$^a$ | 61.90 (51.88)$^a$ |
| Control | 0.00 (2.86) $^c$ | 0.00 (2.86)$^c$ | 0.67 (4.69)$^c$ | 3.00 (9.97)$^c$ |

Each value is mean of three replicates; Values in parenthesis are arc sine transformed values; a - d represents the levels of treatments. That is 'a' = best treatment and 'd'= poor treatment.

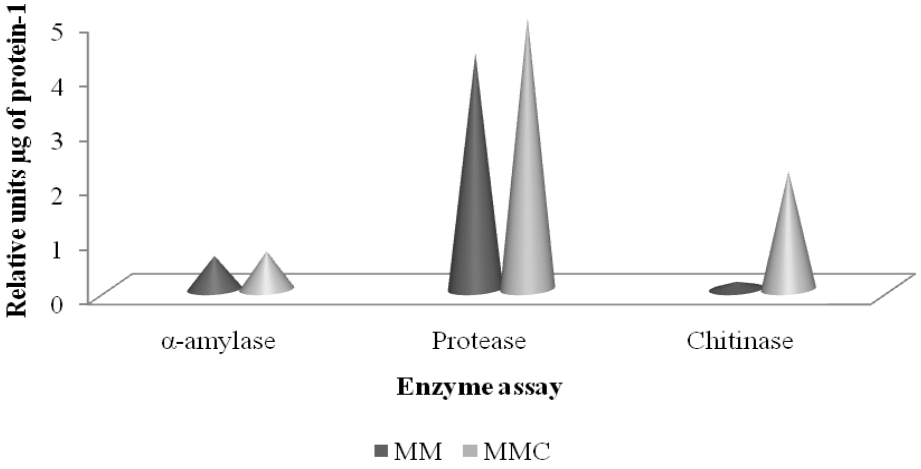


**Figure 1.** Secretome production of *Ifr*$_1$ isolate of *Isaria fumosorosea* response to *Papilio demoleus* exoskeleton.

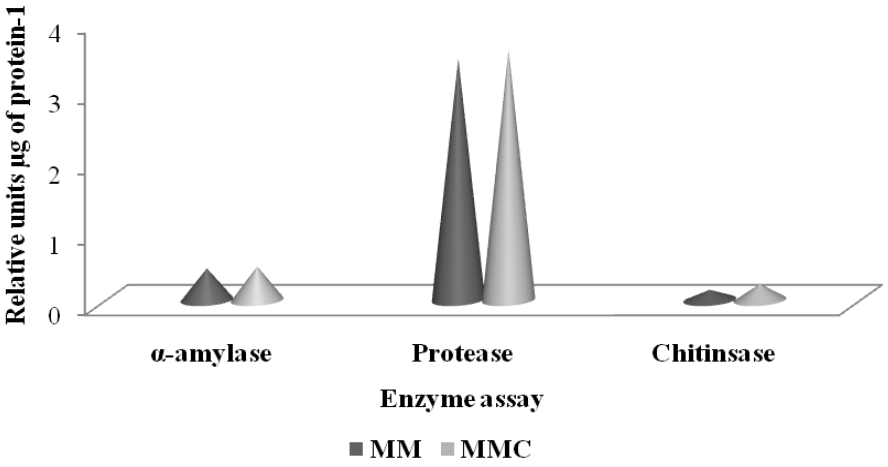


**Figure 2.** Secretome production of *Ifr*$_2$ isolate of *Isaria fumosorosea* response to *Papilio demoleus* exoskeleton.

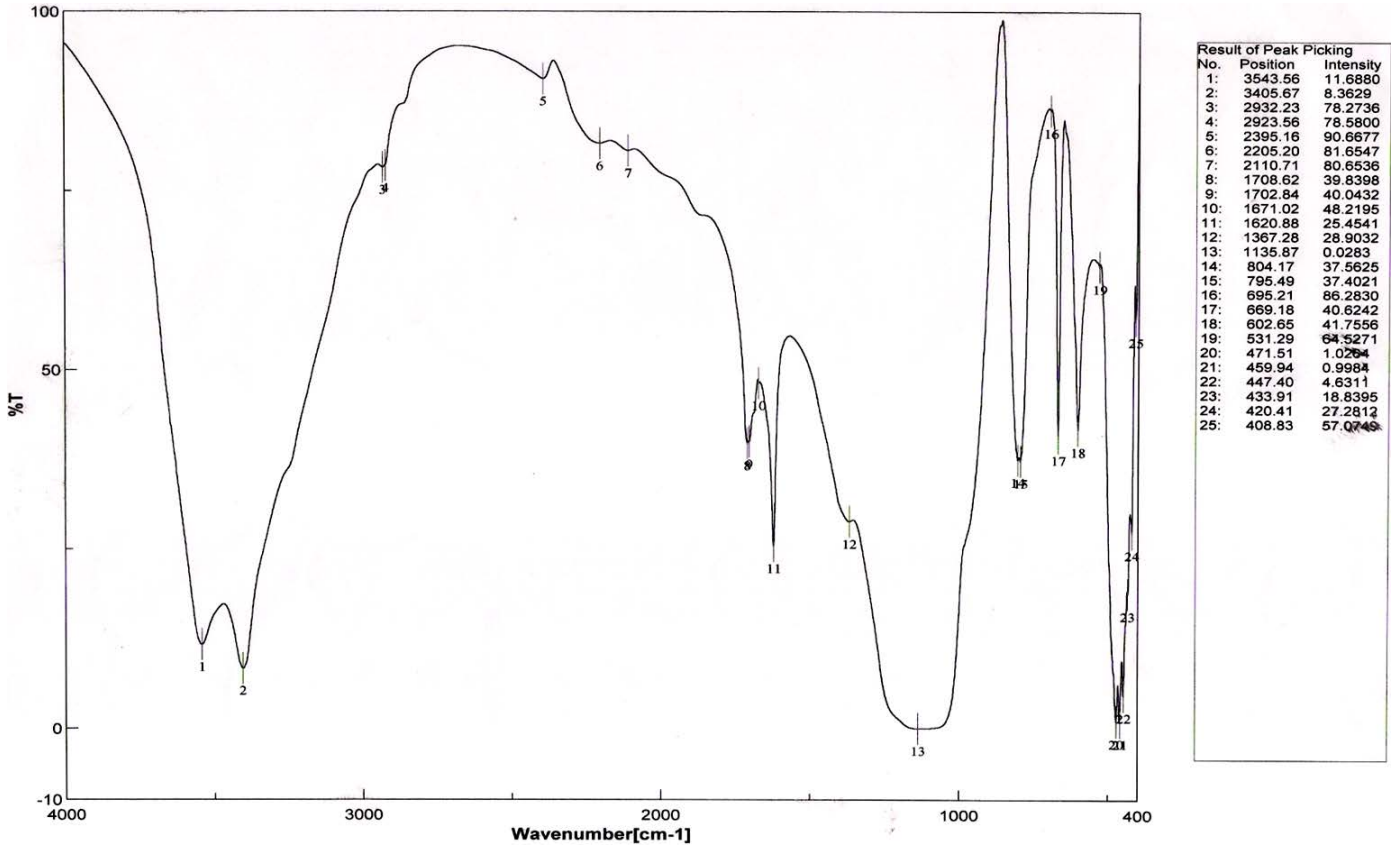


**Figure 3.** Fourier transform infra-red (FTIR) analysis of catalytic triad conserved amino acid of cuticle degrading proteases of *Isaria fumosorosae.*

importance. However, while fungal insecticides have been employed widely in China and to a lesser extent in Eastern Europe as well as in parts of South America, fungi have been little used elsewhere (Charnley, 1997). The virulence of the isolates towards *P. demoleus* was determined to identify the most promising candidate.

In the present study, the tested isolates differed in their pathogenicity towards *P. demoleus* that is the highest mortality caused by the $Ifr_1$ and $Ifr_2$ isolates was 72.23 and 61.90% respectively. The results of present investigations are in accordance with the findings of Nugroho and Ibrahim (2004).

The faster infectivity or shorter time to the broad mite mortality was caused by *P. fumosoroseus* with 2.783 days and gave 50% mortality while *Beauveria bassiana* took 3.349 days and *M. anisopliae* took 4.280 days to cause 50% mortality (Nugroho and Ibrahim, 2004). The reason behind the difference in mortality may be due to the larval susceptibility.

The insect cuticle acts as a barrier for fungal penetration and its thickness increases with every molting so that differences in the susceptibility of different larval instars to entomopathogenic fungi can be explained by their cuticle properties (Boucias and Pendland, 1991). Malsam (1999) reported no differences in the susceptibility of different larval stages to entomopathogens; an increased adult mortality and a decrease in the reproduction caused by *M. anisopliae*. Van De Veire et al. (1996) observed similar susceptibility to *P. fumosoroseus* in all larval stages and adults except for the 2nd stage, which was less susceptible.

The length of the inter-molt period depends upon the environmental conditions and the shorter it gets the less time remains for the fungus to germinate and penetrate. If molting occurs shortly after inoculation the entreating fungus may be removed prior to the colonisation of the insect (Vey and Fargues, 1977; Fargues and Rodriguez-Rueda, 1971).

Hence, the differences in the mortality can be explained by the time of inoculation regarding the remaining period to ecdysis. Presumably, susceptibility of most insects to entomopathogens is related to spore dosage. The speed of kill is influenced by the number of infection propagules in contact with the cuticle.

For most insect/ pathogen combinations a positive correlation between the number of infective spores and mortality by mycosis has been established (Liu et al., 1989; Vestergaard et al. 1995). Additionally, not all areas of the insect cuticle are equally vulnerable to penetration by propagules of entomopathogenic fungi (Butt and Goettel,

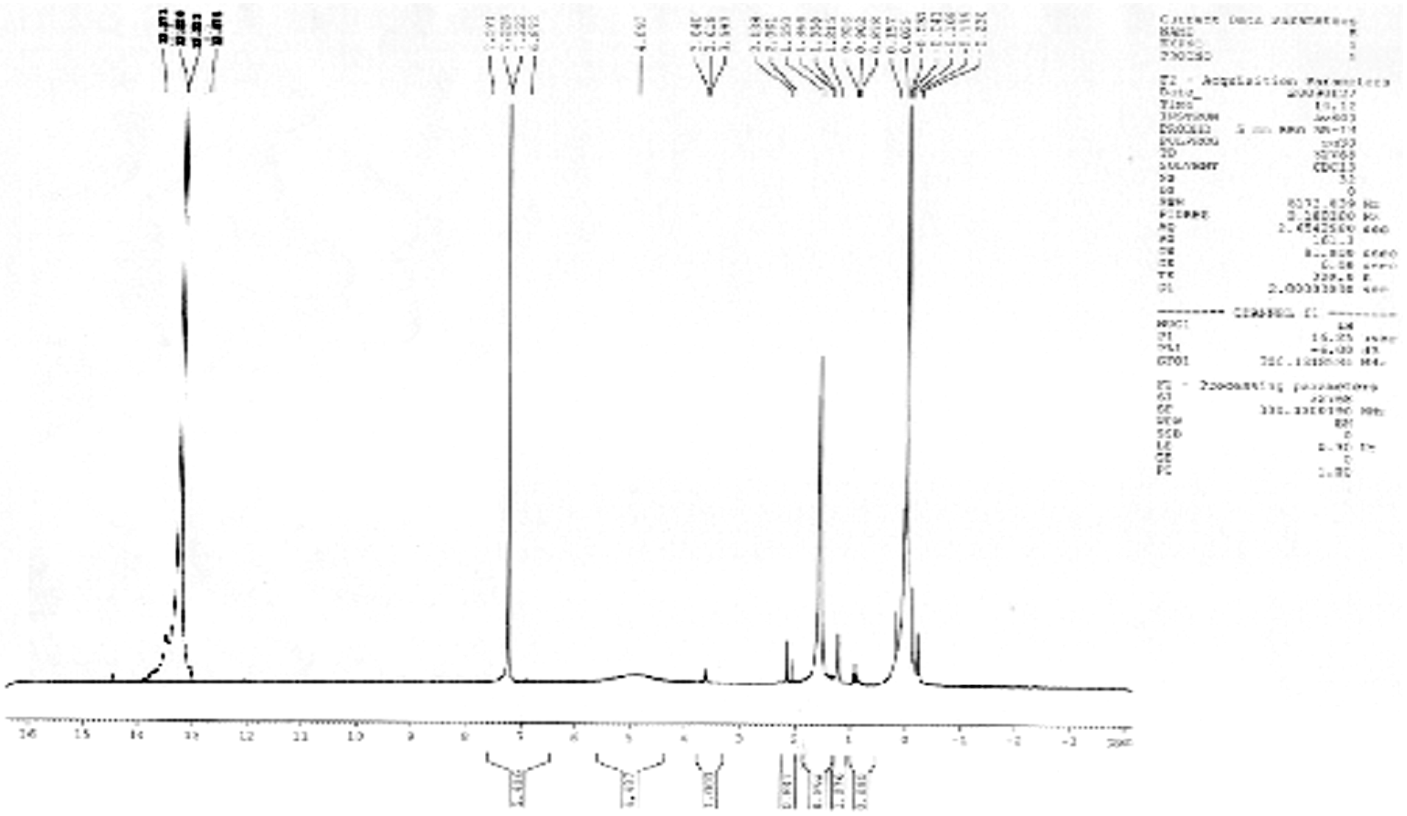

**Figure 4.** $^{1}$H nuclear magnetic resonance (NMR) analysis of catalytic triad conserved amino acid of cuticle degrading proteases of *Isaria fumosorosea.*

2000). The preferential sites of invasion by fungi are often the buccal cavity, the area under the elytra, the inter-segmental folds or spiracles, where locally high humidity promotes germination and the cuticle is nonsclerotised and more easily penetrated (Charnley, 1989; Clarkson and Charnley, 1996; Hajek and St. Leger, 1994; Schabel, 1976). In the present study, it was evident that, spore concentration at $10^{8}$ revealed higher mortality to *P. demoleus* and particularly, *Ifr*$_1$ isolate hed the higher control potential among the isolates and the spore concentration considered and reccommended for the effective mangement of *P. demoleus.*

The present investigation found relatively slight increase in the secretion of protease by the *Ifr*$_1$ in the presence of *P. demoleus* cuticle. The secretion of protease in the presence of cuticle in the present investigation was lower compared to the results found by Murad et al. (2006, 2007), whose reports contained higher hydrolytic enzyme secretion in the presence and absence of *Collasobruchus maculatus* exoskeleton treated with *Metarhizium anisopliae* and *Beauveria bassiana* accordingly.

However the present investigation indicates relatively lower protein secretion by the *Ifr*$_1$ in response to *P. demoleus* exoskeleton than *Ifr*$_2$. The secretions were expected since the unique carbon and nitrogen sources supplied to the fungus were derived from chitin-rich and protein-rich exoskeletons. The proteinases and chitinase clearly indicates the strong support played by the secretomes in insect killing efficiency of the isolate *Ifr*$_1$ than *Ifr*$_2$.

According to the basic structure of amino acids, and the presence of catalytic triad conserved amino acids, cuticle degrading proteases of the *I. fumosorosea* was confirmed with the report from FTIR and $^{1}$H NMR. In the FTIR analysis, the presence OH, $NH_2$, COOH, $CH_2$ and CH groups of the catalytic triad amino acids (Serine, Histidine and Asparagine) were confirmed with peak positions. Presence of serine confirmed by obtaining the peak position at 3543, 3405 and 1135 ($cm^{-1}$) was shared by OH and $NH_2$ group.

The peak position for Carboxyl (COOH), $CH_2$ and CH group were obtained at 2923 ($cm^{-1}$) (OH), 2932 ($cm^{-1}$) (OH), 1708(C-O), 1702 ($cm^{-1}$) (C-O), 1367 ($cm^{-1}$) (O-C), 2932 and 2932 ($cm^{-1}$) accordingly. Besides, peak positions at 3459, 1011, 3405, 2715, 1216, 1224, and 2715 ($cm^{-1}$) was shared by the histidine and asparagine amino acids. Thus, in the present study, not only presence of catalytic triad amino acids was confirmed but also the enzyme activity of the isolate comes under the serine protease criteria. Similarly, the presence of serine was also verified clearly in the $^{1}$H NMR. The peak in the

range 11-14 ppm confirmed the presence of OH group which was the main side chain of the amino acids serine besides, peak at 7.571 ppm (CH), 7.226, 7.222 and 6.873 ppm (CH), 4.893 and 3.640 ppm (CH), 3.619 and 3.597 ppm (CH2), 2.138 and 2.061 ppm ($NH_2$), represents the asparagine and its 2- Pyrrole imidazole ring (7.571 ppm (CH) and 6.873ppm (CH). The peak at the 550, 1.364, 1.338, 1.215, 0.926, 0.902 and 0.878 ppm has the histidine counterparts. Thus, the above information clearly confirm the presence of catalytic triad amino acids in the secreted proteome of *I .fumosorosea* towards *P. demoleus* exoskeleton.

## Conflict of Interests

The author(s) have not declared any conflict of interest.

### REFERENCES


Altre JA, Vandenberg JD, Cantone FA (1999). Pathogenicity of *Paecilomyces fumosoroseus* isolates to diamondback moth, *Plutella xylostella*: correlation with spore size, germination speed, and attachment to cuticle. J. Invertebr. Pathol.73:332- 338.

Birktoft JJ, Blow DM (1972). Structure of alpha-chymotrypsin. J. Mol. Biol. l68:187-240.

Boucias DG, Pendland JC (1991). Attachment of mycopathogens to cuticle. In: Cole, G. T. and Hoch, H. C. (eds.): The fungal spore and disease initiation in plants and animals. Plenum Press, New York, London.

Charnley AK (1989). Mechanisms of fungal pathogenesis in insects. In Whipps JM & Lumsden RD (Eds). The Biotechnology of fungi for improving plant growth. Cambridge University, London, pp. 85-125.

Charnley AK (1997). Entomopathogenic fungi and their role in pest control. In: Wicklow, D. T. and Söderström, B. E. (eds.): The Mycota IV, Environmental and Microbial Relationships. Springer Verlag, Berlin, Germany.

Clarkson J, Screen S, Bailey A, Cobb B, Charnley AK (1998). Fungal pathogenesis in insects. In: Bridge, P., Couteaudier, Y. and Clarkson, J. (eds.): Molecular variability of fungal pathogens. CAB International, Wallingford, UK.

Clarkson JM, Charnley AK (1996). New insights into the mechanisms of fungal pathogenesis in insects. Trends Microbiol. 4:197-203.

Dodson G, Wlodawer A (1998). Catalytic triads and their relatives. Trends Biochem. Sci. 23: 347-352.

Fang QX, Zhou YY, Hu YM, Yang SF, Gong YX (1985). Selection and cultivation of enhanced strains of *Paecilomyces fumosoroseus* to control *Trialeurodes vaporariorum)*. Chin. J. Biol. Cont. 1(2):15-18 (in Chinese).

Fargues J, Rodriguez-Rueda D (1971). Sensibilité des oeufs des Noctuides *Mamestra brassicae* et *Spodoptera littoralis* aux Hyphomycetes *Paecilomyces fumosoroseus* et *Nomuraea rileyi*. C. R. Acad. Sci. 290(1):65-68.

Hajek AE, St. Leger RJ (1994). Interactions between fungal pathogens and insect hosts. Ann. Rev. Entomol. 39(1):293-322.

Haraprasad N, Niranjans SR, Prakash HS, Shetty HS Seema W (2001). *Beauveria bassiana* - A Potential Mycopesticide for the efficient control of coffee Berry Borer, *Hypothenemus hampei* (Ferrari) in India. Biocontrol Sci. Technol. 11:251-260.

Kaijiang L, Roberts DW (1986). The production of destruxins by the entomogenous fungus *Metarhizium anisopliae* var. *major*. J. Invertebr. Pathol. 47(1):120-122.

Lacey LA, Frutos R, Kaya HK Vail P (2001). Insect pathogens as biological control agents: Do they have a future? Biol. Control 21(3):230-248.

Liu SD, Lin SC, Shiau JF (1989). Microbial control of coconut leaf beetle (*Brontispa longissima*) with Green Muscardine fungus, *Metarhizium anisopliae* var. *anisopliae*. J. Invertebr. Pathol. 53(1):307-314.

Malsam O (1999). *Optimierung der Wirksamkeit des entomopathogenen Pilzes Metarhizium anisopliae gegen Weife Fliegen*. PhD thesis, Rhein. Friedrich-Wilhelms-Universitaet, Bonn, Germany.

Murad MA, Laumann RA, Mehta A, Noronha EF Franco OL (2007). Screening and secretomic analysis of enthomopatogenic *Beauveria bassiana* isolates in response to cowpea weevil (*Callosobruchus maculatus*) exoskeleton. Comp. Biochem. Physiol. 145:333-338.

Murad, MA, Laumann RA, Lima A, Sarmento RBC, Noronha EF, Rocha TL, Valadares-Inglis MC, Franco OL (2006). Screening of entomopathogenic *Metarhizium anisopliae* isolates and proteomic analysis of secretion synthesized in response to cowpea weevil (*Callosobruchus maculatus*) exoskeleton. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 142:365-370.

Nugroho I, Ibrahim YB (2004). Laboratory Bioassay of Some Entomopathogenic Fungi against Broad Mite (*Polyphagotarsonemus Latus* Bank). Int. J. Agric. Biol. 6(2):223-225.

Rath AC, Guy PL, Webb WR (1996). *Metarhizium* spore surface antigens are correlated with pathogenicity. Mycol. Res. 100(1): 57-62.

Russell AJ, Fersht AR (1987). Rational modification of enzyme catalysis by engineering surface charge. Nature 328:496-500.

Schabel HG (1976). Oral infection of *Hylobius pales* by *Metarhizium anisopliae*. J. Invertebr. Pathol. 27(1):377-383.

Siezen RJ, Leunissen JAM (1997). Subtilases the superfamily of subtilisin- like serine proteases. Prot. Sci. 6:501-523.

St. Leger RJ, Cooper RM, Charnley AK (1986). Cuticle-degrading enzymes of entomopathogenic fungi: degradation in vitro by enzymes from entomopathogens. J. Invertebr. Pathol. 48:167-177.

St.Leger RJ, Butt TM, Goettel MS, Staples RC Roberts DW (1989). Production *in vitro* of appressoria by the entomopathogenic fungus *Metarhizium anisopliae*. Exp. Mycol. 13(3):274-288.

St.Leger RJ, Durrands PK, Charnley AK Cooper RM (1988). The role of extracellular chymoelastase in the virulence of *Metarhizium anisopliae* for *Manduca sexta*. J. Invertebr. Pathol. 52(1): 285-294.

St.Leger RJS, Charnley AK, Cooper RM (1987). Characterization of cuticle degrading protease produced by the entomopathogen *Metarhizium anisopliae*, Arch. Biochem. Biophys. 253:221-232.

Ulhoa CJ, Peberdy JF (1992). Purification and some properties of the extracellular chitinase produced by *Tichoderma harizianum*. Enzyme Microb. Technol. 14:236-240.

Van De Veire M, Degheele D, Van Lenteren JC (1996). Toxicity of the fungal pathogen *Paecilomyces fumosoroseus,* strain Apopka 97, to the greenhouse whitefly *Trialeurodes vaporariorum* and the parasitoid *Encarsia formosa*, and first results of a control experiment in glasshouse tomatoes. In: *IOBC wprs Bulletin,* Proceedings of the meeting of the working group 'Integrated control in glasshouses', 20.-25. May 1996, Vienna, Austria.

Vestergaard S, Gillespie AT, Butt TM, Schreiter G, Eilenberg J (1995). Pathogenicity of the hyphomycete fungi *Verticillium lecanii* and *Metarhizium anisopliae* to the western flower thrip s, *Frankliniella occidentalis*. Biocontrol. Sci. Technol. 5(2): 185-192.

Vey A, Fargues J (1977). Histological and ultrastructural studies of *Beauveria bassiana* infection in *Leptinotarsa decemlineata* larvae during ecdysis. J. Invertebr. Pathol. 30 (1):207-215.

Vey A, Fargues J, Robert PH (1982). Histological and ultrastructural studies of factors determining the specifity of pathotypes of the fungus *Metarhizium anisopliae* for Scarabeid larvae. Entomophaga 27(4):387-397.

Wraight SP, Carruthers RI, Bradley CA, Jaronski ST, Lacey LA, Wood P, Galaini-Wraight S (1998). Pathogenicity of the entomopathogenic fungi *Paecilomyces* spp. and *Beauveria bassiana* against the silverleaf whitefly, *Bemisia argentifolii*. J. Invertebr. Pathol. 71(1):217-226.

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*Full Length Research Paper*

# Evaluation of oral vaccination of village chickens against newcastle disease with I-2 vaccine coated parboiled cracked maize in Enderta District, Tigray, Ethiopia

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**The study was conducted to assess the suitability of soaked parboiled cracked maize as a carrier of I-2 vaccine for oral immunization of village chickens. Chickens were vaccinated once via ocular route and orally with cracked maize at the second and fifth weeks of the experiment. Post vaccination serum was collected 4, 7, 9 and 11 weeks of the experiment and haemagglutination inhibition test was done to evaluate the antibody titer. The results show that chickens vaccinated via ocular route produce geometric mean $log_2$ HI antibody titers of 5.71 and 5.51 at the fourth and seventh weeks of the experiment, respectively. On the other hand, the antibody titer of chickens vaccinated with coated cracked maize vaccine was 2.54 during the first vaccination; and during the booster vaccination, the titter increased to 2.92. Among the chickens vaccinated via ocular route, 90% (during the first vaccination) and 84.2% (during the booster vaccination) have titer above the protection titer of 3. Similarly, for chickens under cracked maize group, 54 and 74.8% of them were able to produce geometric mean $log_2$ HI antibody titers ≥ 3. The current experimental study indicates that cracked maize can be good candidate carrier of I-2 vaccine virus with easy administration methods for village chickens.**

**Key words:** Antibody, chickens, cracked maize, ocular, haemagglutination inhibition, I-2 vaccine.

## INTRODUCTION

Village chickens are vital for national as well as household economy growth in developing countries. They can play a crucial role in lifting up the nutritional levels and incomes of the rural poor farmers and landless laborer, especially women and children, who are largely responsible for looking after chickens (Melesse et al., 2011; Moges et al.,

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2010; Alders and Spradbrow, 2001). In Ethiopia, chickens are widespread and almost every rural family owns chickens, which provide a valuable source of family protein and income (Tadelle et al., 2003). Rural chickens in Ethiopia represent a significant part of the national economy in general and the rural economy in particular; they contribute 98.5 and 99.2% of the national eggs and chicken meat production, respectively (Tadelle, 1996; Aberra, 2000).

High mortality of chicks due to diseases, malnutrition due to poor quantity and quality of feed, predation, poor housing and insufficient water supply are some of the diverse constraints on village chicken production in Ethiopia (Tadelle and Ogle, 2001; Degumma, 2009). Among the infectious cause of mortality of chicks, Newcastle diseases (ND) is the major one. It causes mortality irrespective of age and sex, which occurs almost any time of the year (Nwanta et al., 2008; Serkalem et al., 2005) and is considered a significant disease throughout the world (Alexander et al., 2004).

The only effective way of controlling ND in epizootic areas is vaccination (Chen and Wang, 2002). However, efforts to vaccinate village chickens would meet with a number of obstacles. Firstly, their free-range life style renders them not amenable to the conventional vaccine delivery methods, namely, aerosol/sprays or drinking water methods. These are only practiced in enclosures for mass vaccination. Secondly, the eye-drop and injection methods are applied individually and demand catching of each chick for vaccination. Thirdly, the conventional vaccine administration demands cold chain. In general, they are designed for intensely reared commercial poultry but are not feasible for village chicken flock in their feral nature (Echeonwu et al., 2007; Nasser et al., 2000).

The advent of heat stable strains of I2 and V4 ND virus vaccines introduced oral delivery through chicken feeds. This presented a feasible method for vaccination of large scattered population of free roaming village chickens as convenient means of protecting them and other poultry in the locality against ND (Spradbrow, 1992). In Ethiopia, parboiled wheat, maize and barley have been identified as suitable carrier of the thermostable vaccine I-2 at laboratory experiment level (Amssalu et al., 2012; Nasser et al., 2000). However the above findings have not been tested at real village set up. Moreover, in a large country like Ethiopia, grain characteristic could vary from region to region.

The main objective of this study is to assess the suitability of soaked parboiled cracked maize as a carrier of thermostable Newcastle disease vaccine $I_2$ for oral immunization of chickens.

## MATERIALS AND METHODS

### Experimental chicken

The experiment was conducted in Enderta District, Didbat Village. The total 210 chicken that comprised chickens of various ages, kept under traditional methods of husbandry from 30 households, were used for the experiment. Households participated based on willingness and were allocated to the three experimental groups: the control, the feed and ocular route each with 70 chickens. Four to ten chickens per house were selected for sampling and marked for re-bleeding. To avoid problem of loss, the whole flock kept by the household were initially vaccinated and bled. Hence in case of lose, death or slaughters by owner, such chickens were easily replaced by other chickens from the flock.

### Vaccination and vaccine application

The current experimental study took 11 experimental weeks. Chickens were vaccinated using feed, parboiled cracked maize two times at the second and fifth experimental weeks. Hence each bird received the booster dose at 21 days from the first date of vaccination. Chickens were vaccinated via ocular route (eye drop) once, at the second week of the experiment. However, chickens in the control group were kept u unvaccinated.

### Eye drop administration

The eye dropper was pre-caliperated (14 ml per 350 dose) and accordingly the vaccine was reconstituted by 14 ml of distilled water. Vaccine was administered within 1 h after preparation and kept cool for the entire vaccination period; chickens received appropriate dose via ocular route. Chickens in the control group were given eye-drops of pure water.

### Administration via soaked parboiled cracked maize

The carrier maize was purchased from the local producer, farmer and washed with tap water. It was then soaked with tab water for two days; where the water was changed every 24 h. After two days, the water was removed and it was sun dried. Then, parboiled grains were prepared as follows. First, the water was boiled and the soaked dried maize was added at a ratio of 1 kg per 3 L of water and left to boil for 10 min. It was then cooled. A lot of water was removed by straining; after which it was sun dried and cracked. Finally, one ampoule (350 doses) of freeze dried I-2 ND vaccine was reconstituted with 175 ml of distilled water (0.5 ml dose per chicken). 0.5 ml (a dose of one chicken) of vaccine suspension was further diluted in 3 ml of clean non chlorinated water and mixed with 10 g of the grain, which is enough to deliver one dose of the vaccine. 10 g vaccine-maize mixture (depending on the flock size) was given to each household identified as part of the study.

### Blood collection for serology

Blood sample (1 ml per bird) was collected using sterile syringe without anticoagulant from brachial vein according to the method described by Alders and Spradbrow (2001). Pre-vaccination blood sample was collected at day one of the experiment (January one) and post vaccination blood was collected every two weeks after the first vaccination and second vaccination at 4, 7, 9 and 11 weeks of the experiment. The blood was allowed to clot over night at room temperature and serum was separated. The sera collected were then stored at -20°C until analyzed.

### Evaluation of immune response

Haemagglutination inhibition was used to evaluate the antibody

**Table 1.** Pre-vaccination geometric mean $log_2$ HI antibody titers.

| Control | Cracked maize | Ocular | Over all mean |
|---|---|---|---|
| 2.26 ±1.052 | 1.70±0.81 | 1.74±1.176 | 1.90±1.05 |

**Table 2.** Post vaccination geometric mean antibody titers of vaccinated chickens and control group.

| Group name | Post vaccination GM±SD HI antibody titer (log2 ) chickens vaccinated by different methods at different experimental week ( N= 70) | | | |
|---|---|---|---|---|
| | Week 4* | Week 7** | Week 9 | Week 11 |
| Control | 1.45±1.051[a] | 1.39±1.046[b] | 1.42±.991[c] | 1.49±.964 [d] |
| Ocular | 5.71±1.972[a] | 5.59±1.861[b] | 4.23±.783[c] | 2.93±.966[d] |
| Parboiled cracked maize | 2.54±1.169[a] | 2.92±1.313[b] | 2.58±1.104[c] | 2.14±.867[d] |

P< 0.001.*two weeks after the first vaccination; **two weeks after the second vaccination (for parboiled cracked maize). Means with the same letters in the same column are significantly different at 0.05 confidence level.

**Table 3.** Percentage of chickens with HI titers greater than or equal to $log_2 2^3$.

| Group name | Number of chickens (%) with HI log2) ≥ 3.0 of all chicks ( N= 70) | | | |
|---|---|---|---|---|
| | Week 4[a] | Week 7[b] | Week 9 | Week 11 |
| Unvaccinated Control | 15 (21) | 14 (20) | 12 (17) | 9 (12.9) |
| Ocular | 63 (90) | 59 (84.2)) | 56 (80) | 42 (60) |
| Parboiled cracked maize | 36 (51.4) | 52 (74.8) | 45 (64.3) | 28 (40) |

[a]Two weeks after the first vaccination; [b]two weeks after the second vaccination (for parboiled cracked maize).

response of vaccinated chickens. Pre- and post-vaccination sera were heat inactivated at 56°C for 30 min. The test was performed following the method described in OIE (2000) manual. Four haem-agglutination (HA) units, 1% chicken erythrocyte suspension and two-fold serial dilutions starting with dilution of 1:2 were used. The antibody level for each serum sample was recorded and was expressed as a log base two. Geometric mean titres (GMT) were calculated for each group.

#### Data analysis

For each variable of interest, the mean value and standard deviation (SD) were determined and classified according to treat-ment groups. The post vaccination mean HI antibody titres were compared by one way analysis of variance (ANOVA). Where the results were significant, Duncan's multiple range tests were done to establish differences in antibody response between pair wise treatments.

## RESULTS

### Base line antibody titer

The overall mean HI titer of chicken was $log_2 2^{1.9}$. All the chickens except those in the control group had HI titre log2<2 before vaccination (Table 1).

### Post vaccination antibody titer

In chickens vaccinated with vaccine coated soaked cracked maize, post vaccination geometric mean $log_2$ HI antibody titer at the 4th week, two weeks after the first vaccination, was 2.54. After the booster dose, at the seventh week, antibody titer of vaccinated chickens was raised to 2.92; whereas the mean $log_2$ HI antibody titer of 5.71 and 5.59 was recorded in chickens vaccinated via ocular route at the fourth and seventh weeks of the experiment, respectively (Table 2).

The antibody titer vaccinated chickens slightly dropped at ninth week of the experiment. The chickens vaccinated via ocular methods and vaccine coated carrier maize showed mean $log_2$ HI titer of 4.23 and 2.58, respectively. As indicated in Table 2, at eleventh week, a further drop in the antibody titer was also noticed. Statistically significant difference was observed among all groups throughout the weeks of the experiment (p = 0.0001).

### Percentage of chickens with protective HI titers

Two weeks after the first vaccination, at fourth week, 90% of chicken vaccinated via ocular method, were able to produce the protective mean $log_2$ HI titer ≥ 3. However,

only 51.4% of chickens under parboiled cracked maize were able to produce above the protective titer. The least percentage of HI titer ≥ 3 was recorded in unvaccinated chickens (Table 3).

Following two weeks after administration of the booster dose, at seventh week, 74.8% of chickens vaccinated with coated cracked maize developed HI titer above the protective level. At this time, 84.2% of chickens vaccinated via ocular route have developed the protective titer. At the ninth and eleventh weeks, the protection percentage decreased in all groups. At the eleventh week, 40% of the chickens vaccinated using cracked maize developed mean $\log_2$ HI titer ≥ 3.

## DISCUSSION

The main objective of this study is to compare the suitability of soaked parboiled cracked maize as carrier for oral delivery of thermostable $I_2$ Newcastle disease vaccine. A total of 210 chicks owned by 30 households were used for the trials. The current study chickens vaccinated via ocular methods have developed higher, protective mean antibody levels than food vaccination. This observation is in line with previous studies by Spradbrow (1992) and Tu et al. (1998). At the fourth and eleven weeks of the experiment, 90 and 60% of chickens have protective titer respectively. The current finding is in agreement with the reports of Illango et al. (2008) and Spradbrow (2001). However, we observed that vaccination of individual chicken via ocular route was difficult because of their feral nature, as it required catching of each bird. This observation has also been reported by Echeonwu et al. (2007). Therefore, this method of vaccination is not convenient for applying vaccine at larger scale for village chickens.

Oral application of vaccine using carrier feed has been mentioned as feasible method for vaccination of large scattered population of free roaming village chickens and convenient means of protecting them (Spradbrow, 1993). In this study, the mean log2 HI titer was 2.54 two weeks after the first vaccination and 2.92, at seventh week after the second vaccination. The overall population with protective antibody titer ≥ $\log_2 2^3$ after first and booster dose vaccination was 50 and 74.8% in the vaccinated chickens, respectively. Amssalu et al. (2012) have reported higher mean log2 HI titer of 7.1 with 100% protection after third vaccination using the same grain. Moreover, Echeonwu et al. (2008) reported soaked parboiled carked maize as good carrier for the vaccine with higher antibody titer than the present finding. However, all the previous report was conducted at the laboratory condition, whereas the current study was conducted under field condition with free roaming chickens. Different reports have indicated that test results on the same grain in laboratory and field conditions differ. This could be one reason for lower HI titer in the present finding (Spradbrow, 2001; Aini, 1990; Rushton, 1995).

In addition, in the current study, chickens received single booster dose. However, as described by Oakeley (2000), for feed base vaccine to give effective protection, it requires at least two booster doses. The mean log2 HI titer of 2.14 at eleventh week in the present finding also indicates the importance of the second boosted dose. Although, it needs second booster dose after short period, according to Oakeley (2000), the 74.8% protection level at seventh week, after booster dose in current study is an acceptable level of protection to control Newcastle disease in village chickens. In addition, Alexander et al. (2004) have indicated that high titer guarantees high survivor, but low antibody titer does not mean low survivor because of the importance of cellular immunity. This fact can also increase the protection level of the current finding.

In conclusion, soaked parboiled cracked maize was found to be good candidate carrier for I2 vaccine virus with easy administration methods for village chickens. However, it needs further improvement. Further research should be conducted on same or different grains from different agro-ecology and soil characteristics.

## Conflict of Interests

The author(s) have not declared any conflict of interest.

## REFERENCES

Aberra M (2000). Comparative studies on performance and physiological responses of Ethiopian indigenous ('Angete-melata') chicken and their F1 crosses to long term heat stress. PhD thesis. Martin-Luther University, Halle-Wittenberg, Berlin, Germany, 182 pp

Aini I (1990): Control of poultry diseases in Asia by vaccination. World Poult. Sci. J. 46:125-132

Alders RG, Spradbrow PB (2001). Control of Newcastle Disease in village chickens: A Field Manual. ACIAR, Canberra, Australia. pp. 1-7.

Alexander DJ, Bell J G, Alders RG (2004). Technology Review: Newcastle Disease. FAO, Animal Production and Health Paper, Rome, 1-26

Amssalu K, Dugumma R, Sorri T, Asfaw Y, Gelaye E (2012): Onsation evaluation of different grains as a carrier for thermostable newcastle disease vacccine (I2), Debreziet, Ethiopia. Proc. 13th International Symposium on Vet. Epid. and Economics, Belgium, Netherlands, Session 67 - Epidemiology in veterinary practice: poultry. p. 256.

Chen JP, Wang CH (2002). Clinical and experimental evidence for the transmission of Newcastle disease virus through egg. Avian Dis. 46: 461-465

Degumma R (2009). Understanding the role of indigenous chickens during the long walk to food security in Ethiopia. Livestock Research for Rural Development, 16 (9) accessed, January 19, 2014, from http://www.lrrd.org/lrrd16/2/deme1602.htm

Echeonwu BC, Ngele MB, Echeonwu GON, Joannis TM, Onovoh EM, Paul G (2008). Response of chickens to oral vaccination with Newcastle disease virus vaccine strain I2 coated on maize offal. Afr. J. Biotechnol. 7 (10): 1594-1599.

Echeonwu GON, Iroegbu CU, Echeonwu BC, Ngene A, Olabode AO, Okeke OI, Ndako J, Paul G, Onovoh EM, Junaid SA, Nwankiti O (2007). Delivery of thermostable Newcastle disease (ND) vaccine to chickens with broken millet grains as the vehicle. Afr. J. Biotechnol. 6 (23): 2694-2699.

Illango J, Mukiibi-Muka G, Abila PP, Musisi G, Etoori A (2008). The value of the Newcastle disease I-2 thermostable vaccine use in the rural free-range poultry management system in Uganda. Livest. Res. Rural Dev. 20:9.

Melesse A, Tiruneh W, Negesse T (2011). Effects of feeding Moringa stenopetala leaf meal on nutrient intake and growth performance of Rhode Island Red chicks under tropical climate. Trop. Subtrop. Agroecosyst. 14:485-492.

Moges F, Melesse A, Dessie T (2010): Assessment of village chicken production system and evaluation of the productive and reproductive performance of local chicken ecotype in Bure district, North West Ethiopia. Afr. J. Agric. Res. 5(13):1739-1748.

Nasser M, Lohr JE, Mebratu GY, Zessin KH, Baumann MPO, Ademe Z (2000). Oral Newcastle disease vaccination trials in Ethiopia. Avian Pathol. 29(1):27-34.

Nwanta J, Egege ASC, Alli J, Balogun K, Ezema ws (2008). Evaluation of prevalence and seasonality of Newcastle disease in chicken in Kaduna, Nigeria. World Poult. Sci. J. 64:416-425.

Oakeley RD (2000). The limitation of a feed/water based heat-stable vaccine delivery system for Newcastle disease control strategies for backyard poultry flocks in Sub-Saharan Africa. Prev. Vet. Med. 47: 271-279

OIE (2000). Newcastle Disease, Manual of standard for diagnostic tests and vaccines. Office International des Epizootics, (OIE), Paris: 221-232

Rushton J (1995). Assistance to rural women in protecting their chicken flocks against Newcastel disease. Consultancy report on rural poultry production- socio-Economy, FAO, Rome: 55-56

Serkalem T, Hagos A, Zeleke A (2005). Seroprevalence Study of Newcastle Disease in Local Chickens in Central Ethiopia. Int. J. Appl. Res. in Vet. Med. 3(1):45-53.

Spradbrow PB (1992). A review of the use of food carriers for the delivery of oral Newcastle disease vaccine. In: Spradbrow, P. B. (ed.): Newcastle Disease in village chicken.

Spradbrow PB (1993). Newcastle Disease in Village Chickens. Poult. Sci. Rev. 5: 57-96.

Spradbrow PB (2001). Thermostable Newcastle disease vaccines. In: Alders, R.G. and Spradbrow, P. B. (ed.). SADC planning workshop on Newcastle disease control in village chickens: organized by ACIAR. Melbourne: Brown Prior Aderson: 61-66.

Tadelle D (1996). Studies on village poultry production systems in the central highlands of Ethiopia. MSc Thesis. Swedish Uni. Agri. Sci. 70 pp.

Tadelle D, Million T, Alemu A, Peters KJ (2003). Village chicken production systems in Ethiopia: Use patterns and performance valuation and chicken products and socio-economic functions of chicken. Livestock Research for Rural Development (15)1. (Available from http://www.lrrd.org/ lrrd15/1/tadeb151.htm) (Accessed on 1 September 2013).

Tadelle D, Ogle B (2001). Village poultry production system in the central highlands of Ethiopia. Trop. Anim. Health Prod. 33(6):521–537.

Tu TD, Phuc KV, Dinh NTK, Quoc DN, Spradbrow PB (1998). Vietnamese trials with a thermostable Newcastle disease vaccine (strain I-2) in experimental and village chickens. Prev. Vet. Med. 34: 205-214.

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