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Demutagenic potential of *Brassica oleracea* and its associated lactic acid bacteria

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Abstract

Cancer chemoprevention is a major cancer preventive strategy that utilizes naturally occurring dietary phytochemicals to inhibit the malignant transformation of initiated cells. The consumption of cruciferous vegetables is claimed to be important in the prevention of cancerous diseases, and thus, the determination of the antimutagenicity of the edible vegetable, *Brassica oleracea* and its associated lactic acid bacteria is quite significant. All fresh cabbage samples yielded positive bacterial growth MRS agar plates while the rotten samples had none. Forty-two isolates were then presumptively identified as lactic acid bacteria after being subjected to cultural, morphological, and biochemical characterization. Potential demutagenic properties of suspensions of cabbage and lactic acid bacteria were assessed using the Ames test. No mutagenicity was found in the 10 cabbage suspensions to *Salmonella typhimurium* TA98 as well as that of the 42 lactic acid bacterial suspensions.

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Introduction

Brassica oleracea, commonly known as cabbage, is a popular vegetable and versatile ingredient in Philippine gastronomic art. Aside from being a culinary staple, cabbage has long been known for its medicinal and healing properties (Sun *et al.*, 2013). Documented traditional uses of raw cabbage juice are for curing ulcers, infections, gastric disorders, and is also considered to be a good cleanser and detoxifier (Patel and Prajapati, 2012). Because of its folkloric claims, cabbage has been called "poor man's medicine chest" and "doctor of the poor" (Woodman, 2003).

In modern medicine, cabbage is till touted for its medicinal benefits as extensive modern research largely confirms its ancient use in folk medicine (Dinova and Kostov, 2012; Tse and Eslick, 2014). Cabbage belongs to the family of cruciferous vegetables, which are regarded as particularly rich source of anticarcinogenic phytochemicals. Isolated substances found in crucifers displayed activities preventing cancer development at several stages and contain precursors of all five most promising natural chemopreventive agents (Bartoszek *et al.*, 2005). The indole-3-carbinol (I3C) compound found in cabbages is considered to be a powerful cancer fighter used in combination with chemotherapy (Wu *et al.*, 2010).

Anticarcinogenic properties of cabbage do not rely solely on compounds associated with this cruciferous vegetable. Cabbage leaves are a good host of lactic acid bacteria (LAB) which facilitates fermentation. This process increases the numbers of LAB enormously, thus, when cabbages begin to decompose, the lactic acid bacteria increase greatly (Touret *et al.*, 2018). The products of this microbial fermentation have been extensively studied for their anticarcinogenic activities. Successful researches of lactic acid bacteria (LAB) reflect their potential application as anticarcinogens or antimutagens (Boubekri and Ohta, 1996).

Making use of cheap, affordable, and readily available vegetables with probable low mutagenic potential is a way of promoting its cultivation and inclusion of these vegetables in the regular diet of every individual (Antwerpen, 1993). Thus, this study generally aimed to test antimutagenic potential of cabbage suspension and lactic bacteria isolated from fresh and rotting cabbage collected from the local marketing place of Iligan City, Philippines.

Materials and methods

Collection and processing of Brassica oleracea

Twenty *Brassica oleracea* were bought from a local market, were placed in individual sterile plastic bags and were immediately brought to the laboratory for further processing. Samples of *B. oleracea* were crushed and homogenized and one gram of each macerated sample was suspended into sterile screw-capped tubes with ten milliliter sterile distilled water, shaken vigorously for a minute, and then serially diluted.

Isolation of lactic acid bacteria from B. oleracea samples

One hundred microliters form each dilution tubes was pipetted and spread-plated onto de Man, Rogosa, and Sharpe (MRS) agar plates. These were incubated anaerobically for 48 hours and then observed for colonial growth. Basic characteristics of colony morphology were noted: size (in millimeters), colony shape (circular, irregular, punctiform); margin (entire, undulate, lobate, filamentous); elevation (flat, raised, convex, umbonate); and color (white, cream, translucent, shiny) (American Type Culture Collection, 2015). Three representative colonies from each plate were restreaked unto MRS agar twice to ensure pure colony culture. Purified isolates were then stored in vials for maintenance.

Determination of cellular morphology

Twenty-four hour-old bacterial cultures were morphologically characterized with the aid of Gram-staining technique (American Type Culture Collection, 2015). The stained smears were observed under the oil immersion objective (OIO, 1000x) of the microscope. Bacterial cells with the same cellular morphology were examined closely, taking particular notice to cells with rod-like shape, arranged singly,

and was stained blue to violet.

Biochemical characterization of bacterial isolates from B. oleracea

Pure bacterial cultures were subjected to biochemical tests to ascertain its presumptive identification.

Nitrate reduction test: Each bacterial culture was inoculated onto screw-capped tubes with ten milliliter of nitrate broth and inverted Durham tube. Tubes were then incubated at room temperature for 24 hours then examined for possible gas formation in the Durham tube. Presence of bubbles is indicative that the microorganism is not a fermenter. Eight drops of sulfanilic acid and α-naphthylamine were added to the tube negative with gas formation. Zinc powder was added to the tubes that did not turn red. If the tube has changed its color to red upon the addition of powder, the result is negative, otherwise if tube remained unchanged, the result is positive (Buxton, 2011).

Indole Test: Isolates were then stab-inoculated onto Sulfur-indole Motility (SIM) medium. Incubation followed at ambient room temperature for 24-48 hours. After incubation, Kovac's reagent was added to the tubes and was observed for the formation of red color in the reagent layer. Formation of red color was indicative of a positive result implying the presence of the enzyme tryptophanase, responsible for cleaving the tryptophan thus producing indole, pyruvic acid and ammonia (McFaddin, 1980). The absence of color change in the reagent layer implied that the test organisms were indole negative (Leboffe and Pierce, 2006).

Determination of antimutagenic potential of cabbage suspension and isolated presumptively identified lactic acid bacteria

Ames test was employed to determine the ability of the cabbage suspensions and isolated lactic acid bacteria to cause reverse mutations in histidine negative strains of *Salmonella typhimurium*TA98. Bacterial suspension of the control microorganism was prepared in ten milliliter of distilled water following the 0.5 McFarland Turbidity Standard then incubated for twenty minutes at 37°C. After incubation, three microliters of the suspension was inoculated and spread-plated onto minimal glucose agar (MGA). This will serve as the control plate. Another batch of *S. typhimurium* suspensions were prepared with 100 microliters of cabbage extract or lactic acid bacterial suspensions. Three microliters of the mixture from each tube were MGA inoculated and spread-plated onto m plates, these served as the experimental plates. Both the experimental and control set-ups were incubated for 48 hours at 37°C, Number of colony-forming units (CFUs) were counted and determined for each plate. (Vijay *et al.*, 2018).

Percent revertant of each LAB isolate was calculated using the following formula:

$$\text{Percent revertant} = \left(\frac{\text{Ave. number of isolates (experimental)}}{\text{Number of colonies (control plate)}} \times 100\right) - 100$$

Results and discussion

Isolation and characterization of bacterial strains from Brassica oleracea

All cabbage samples had smooth leaf surfaces, seventeen (85%) had round-shaped heads and thirteen (65%) were pale green in color.

The rotten cabbages, on the other hand, were grayish brown with dark spots, has withered leaves, and foul odor. Both fresh and rotten leaves of cabbage samples were used for the detection of lactic acid bacteria.

MRS plates inoculated with extracts from rotten cabbage samples have been detected to have negative growth of bacterial colonies. This may be due to the molds that grow on the withered leaves of cabbage during decomposition stage.

On the other hand, MRS plates inoculated with the fresh cabbage extracts have yielded positive growth of bacterial colonies. Forty-two (42) bacterial isolates from the plates were identified and characterized. Isolated colonies were observed and characterized for their size, shape, margin, elevation and color (Table 1).

Table 1. Colonial characterization of isolates.

Colonial characteristics	Number of isolates
A – circular, entire, convex, cream	19
B – circular, entire, convex, white	14
C – punctiform, entire, convex, translucent	1
D – circular, entire, umbonate, cream	2
E – punctiform, entire, convex, white	1
F – punctiform, entire, convex, cream	2
G – circular, entire, convex, translucent	1
H – filamentous, filamentous, raised, white	1
I – filamentous, filamentous, raised, cream	1

Nine major characteristics were observed and the most predominant bacterial isolate (45%) have the morphology of having circular shape, entire margin, convex elevation and cream color. All isolates have measurements ranging from one to three millimeters (1-3 mm).

Typical colony characteristics of *Lactobacillus* are usually non-pigmented being white to light gray and sometimes opaque and creamy in appearance. Various strains vary from different species; some grew with filamentous edges and seldom raised but commonly appeared circular, rough and with entire margin (Bergey's Manual of Determinative Bacteriology, 1994).

These colonies that have been observed to grow in the MRS plates had similar colonial characteristics of lactic acid bacteria as described in the Bergey's Manual of Determinative Bacteriology, however, further microbiological tests were employed to ensure its presumptive identity.

Cellular morphology characterization: gram staining

Gram staining and microscopy were employed to classify and differentiate twenty-four hour old colonies according to gram stain reaction. Eighty-one percent of the bacterial isolates were examined to be gram-positive.

The shape of the isolated colonies were studied and classified as coccobacillus (55%) and bacillus (45%). Bacterial Isolates were noted to occur singly or in clusters.

Lactic acid bacteria have the characteristics of being gram-positive and vary in morphology from long, slender rods to short coccobacilli, which frequently form chains but seldom in singles (Bergey's Manual of Determinative Bacteriology, 1994).

Based on the characterization of isolates, were the presumptive identified lactic acid bacteria eighty-one percent (81%) which were gram positive and bacillus in shape.

Biochemical characterization: nitrate reduction and indole tests

Forty-two (42) bacterial isolates were observed to be indole negative which implied that the strains were not able to convert the tryptophan into pyruvate and indole (Mac Williams, 2009). This was evidenced by unchanged color upon addition of Kovac's reagent. Indole positive organism should react with the medium turning Kovac's reagent to red (Zahoor *et al.,* 2003).

Furthermore twenty-five (25) bacterial isolates (60%) were determined to be incapable of reducing nitrate as evidenced by the change of color to red upon the addition of zinc powder to the inoculated tubes.

Nitrate broth was used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase.

Table 2. Revertant *S. typhimurium* colonies per cabbage suspension.

Cabbage suspension	Average number of revertant colonies	Percent decrease (%)
1	2	98
2	22	83
3	17	87
4	1	99
5	8	94
6	41	68
7	30	77
8	18	86
9	1	99
10	1	99

Based on the data gathered, twenty isolates cultured from MRS agar possessed the characteristics of gram positive rod-shaped cellular morphology and physiological traits of being unable to reduce nitrate and inability to break down tryptophan. However, there were fourteen isolates having the same features but these fourteen exhibited positive result for the nitrate reduction test. Ttherefore, as a result of the totality of the tests employed, thirty-four isolates (81%) were presumptively-identified as lactic acid bacteria.

Mutagenic potential of cabbage and lactic acid bacteria

Ames test is a rapid, inexpensive means of using specific bacteria to evaluate the mutagenic properties of potential carcinogens (Leboffe and Pierce, 2006). In this study, *Salmonella typhimurium* TA98was used as the test bacteria while cabbage extracts and suspensions of presumptively identified lactic acid bacteria were used as test agents verified for potential mutagenic or carcinogenic properties. Lesser growth of test bacteria means less mutation and less probability of mutagenic and carcinogenic properties (Falck, 1987).

Three MGA plates inoculated with *S. typhimurium* alone yielded an average colony growth of 129. On the other hand, experimental MGA plates, with both *S. typhimurium* and macerated cabbage suspensions, yielded an average revertant colonies of 1-41 (Table 2). The results obtained were significantly lower (68%-98% decrease) than that of the control plates. This implies that the cabbage suspensions have no mutagenic potential and is safe for human consumption. This conforms to the study of Gautam *et al.,* (2016) where the antimutagenic activity of many vegetable juices were evaluated. All cruciferous vegetables including cabbage showed strong to moderate antimutagenic activities.

There is a vast array of documented scientific evidences endorsing beneficial role of fruits and vegetables in the prevention as well as treatment of different diseases due to their biologically active substances (Kris-Etherton *et al.*, 2002; Paganga *et al.*, 1999; Proteggente *et al.*, 2002) and cabbage has been identified to have a substantial potential for human cancer chemoprevention (Abdull Razis and Noor, 2013).

Another Ames test was performed using the lactic acid bacteria suspensions as the test agents examined for possible mutagenic or carcinogenic ability. All culture plates treated with the lactic acid bacterial suspensions showed lesser *S. typhimurium* growth than the control plate that has no LAB suspension added which ranged from 18% (95 colonies) to 90% (13 colonies) percent decrease (as shown in Table 3).

The same explanation could be deduced, the bacterial isolates were nonmutagenic which means it could not cause cell mutation that may cause cellular malfunctioning resulting to cancer.

Table 3. Revertant *S. typhimurium* colonies per lactic acid bacterial suspension.

Isolate	Average number of revertant colonies	Percent decrease (%)
1	54	58
2	64	50
3	64	50
4	29	77
5	71	45
6	75	42
7	61	53
8	84	35
9	50	61
10	75	42
11	59	54
12	45	65
12	65	50
14	44	66
15	65	50
16	60	53
17	95	27
18	98	26
19	95	26
20	74	43
21	98	24
22	97	25
23	66	49
24	71	45
25	79	39
26	61	53
27	76	41
28	95	26
29	69	47
30	13	90
31	26	80
32	24	82
33	73	43
34	88	32
35	106	17
36	95	18
37	100	23
38	69	47
39	66	49
40	79	39
41	57	56
42	98	24

Probiotic bacteria (mostly lactic acid bacteria) and their products of fermentation are claimed to provide antimutagenic actions. Mechanism of the antimutagenic activity has not yet been clearly understood. However, the possible mechanism of antimutagenicity could be attributed to the binding of mutagens to microbial cells which exhibited deactivation upon the binding to the cell (Thapa and Zhang, 2008). Thus, the results of the study supports the belief that consumption of vegetables such as cabbages may contribute significantly to the improvement of human health as a natural source of Therapeutic agents (Ambrosone and Tang, 2009).

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