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EFFECTIVENESS OF BLEACH IN THE ELIMINATION OF BACTERIAL FLORA ON LETTUCES GROWN IN SOUTH BENIN

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ABSTRACT

Lettuce, a vegetable often eaten raw, is one of the most cultivated leafy vegetables in South Benin. Its consumption is growing and associated with the problems of microbiological contamination. The specific objective of this study is to determine the concentration of bleach and the time required to eliminate on lettuces almost all the total mesophilic flora, flora of Escherichia coli and Staphylococcus with coagulase-positive. After a preliminary survey on the products used by the populations of South Benin for the disinfection of lettuce, the bleach was selected and then tested in the laboratory. The experiment consisted in determining, for each bacterial species, the number of residual bacteria in samples of lettuce that had remained in the bleach at different concentrations and during different contact times namely 5 min, 10 min and 15 min. Bleach at 12° chl (3.6% active chlorine), purchased commercially, was diluted to 1/100th (0.12° chl), 1/200th (0.06° chl) and 1/500th (0.024° chl) with tap water respectively in three sterilized stainless-steel containers. The results of our study suggest that bleach at 0.06° chl for 15 min would be effective in substantially removing both the flora of Escherichia coli and coagulase-positive Staphylococcus which are germs harmful to human health.

Keywords: Bleach - bacterial flora - lettuce

INTRODUCTION

In general, in Benin, lettuce is eaten raw in salads. Like most leafy vegetables, it is widely grown from poultry manure, which is widely used in market gardening in south Benin because of their high availability and richness in nitrogen (Ahouangninou et *al*., 2013); they are mostly applied directly in the fields without any prior treatment. However, these manures, often rich in pathogenic microorganisms (Puterflam et *al.*, 2005), may reflect the poor health of the poultry from which they are derived. In addition,studies have shown that the use of raw poultry manure in market gardening has an impact on both the microbiological and chemical quality of the vegetables produced (Métras, 2003; Kouakou et *al*., 2005; Dougnon, 2013). Also, according to the CECMA (Quebec), "fresh vegetables and fruits can be vectors of pathogenic microorganisms from organic fertilizers" (CQIASA, 2009). Thus, aware of the health risks associated with the consumption of vegetables produced with poultry manure, the populations of South Benin use various products to disinfect lettuces before consuming them.

There are two main families of disinfectants: antiseptics, intended to disinfect living tissues (skin, mucous membranes, wounds) and disinfectants, used for inert objects and surfaces (floors, surfaces, medical devices, etc.) (Massicotte, 2009; Slama and Djemil, 2004).

No disinfection is possible if it has not been preceded by effective cleaning. The purpose of cleaning is to remove dirt (greases, sugars, limescale, etc.) as they can interfere with the effectiveness of the disinfectant (Massicotte, 2009). According to Widmer and Dettenkofer (2002), cleaning would reduce the environmental burden of microorganisms by 80%; cleaning and disinfection are therefore complementary. Most of the substances used for cleaning are detergents (Leyral and Vierling, 2001). Thus, there is a difference between a detergent and a disinfectant. A detergent dislodges organisms or dirt that adhere to a surface; the fact that an organism is detached from its support increases the contact surface with a disinfectant and consequently the effectiveness of the latter. A disinfectant attacks the constituents of microorganisms (Massicotte, 2009), it is able to inhibit their growth or kill them. According to Leyral and Vierling (2001), a good disinfectant has some or all of the following qualities:

- persistent action (afterglow);
- the widest possible spectrum of activity;
- it acts in low doses;
- it is without corrosive action on the material;
- it does not leave residues after rinsing;
- absence of bacterial resistance phenomenon.

Bleach is a solution of sodium hypochlorite (NaClO) and sodium chloride (NaCl), the concentration of which is expressed in chlorometric degree (° chl) or as a percentage of active chlorine. From the nineteenth century, sodium hypochlorite was used as a disinfectant as well as for the treatment of drinking water. Its disinfectant power comes from its oxidizing properties due to the presence of the hypochlorite ion ClO^- which attacks the cytoplasmic membrane (Joffin and Chevalier, 2002). Sodium hypochlorite is a sodium salt of hypochlorous acid HOCl. In solution, sodium hypochlorite dissociates into sodium (Na^{+}) and hypochlorite (ClO^-) ions ($NaClO \rightarrow Na^+ + ClO^-$).

Chlorine reacts with organic matter (blood, saliva, etc.), which reduces its bactericidal power. Sodium hypochlorite should therefore be used on surfaces free of soiling. The bactericidal effect of the product is also influenced by pH, concentration, temperature, the presence of ammonia and the addition of other halogens (Paris-Nord, C. C., 2000). Sodium hypochlorite has no residual effect; since the product has no afterglow effect, the disinfected surface may be contaminated a few seconds later. In addition, there are still no precise data on the toxicity threshold of bleach.

The general objective of this research is to study the effectiveness of bleach on the bacterial flora of lettuces before their consumption. Specifically, it was a question of:

- explorer the practices of disinfection of lettuce by the populations of South Benin;
- determine the concentration of bleach and the time required to remove almost all of the total mesophilic flora, *Escherichia coli* and *Staphylococcus* coagulase+ flora onfresh lettuce.

MATERIALS AND METHODS

1. Survey on the practices of disinfection oflettuce by the populations of South Benin

The survey was carried out in particular in the communes of Cotonou and Abomey-Calavi in order to determine the products used for the disinfection of lettuces and their mode of use by the populations of South Benin. The participants of the survey are consumers of lettuces targeted in markets that have been chosen according to their level of attendance, these include the markets of Calavi-Tokpa, Cococodji, Godomey in the municipality of Abomey-Calavi, then Saint-Michel, Ganhi and Gbe gamey in the commune of Cotonou. A simply incidental sample of 79 consumers was interviewed using an interview guide. This preliminary investigation confirmed our choice of bleach in the conduct of this study.

2. Bleach lettuce disinfection and microbiological analyses

We tested in the laboratory the effect of bleach on the bacterial flora of lettuces for consumption.' Specifically, we determined the number of residual bacteria, including total flora, *Escherichia coli* and *Staphylococcus* coagulase positive, in samples of lettuces that had stayed in bleach at different concentrations and during different contact times (5 min, 10 min and 15 min), l e contact time being the time it takes for a disinfectant to inactivate an organism (Massicotte, 2009).

Bleach at 12° chl (3.6% of active hplore), purchased commercially, was diluted to 1/100th (0.12° chl), 1/200th (0.06° chl) and 1/500th (0.024° chl) by taking 10 ml, 5 ml and 2 ml respectively from three sterilized stainless-steel containers and then supplementing these volumes up to 1 liter with tap water. A sample of about 500 g of fresh lettuce was purchased at the market in sterile sachets, stored in an insulating box at 4°C and then transported to the laboratory. First, dix grams (10 g) of this sample were taken and analysed for the enumeration of the bacteria mentioned above in order to determine the initial state of the bacterial flora on these lettuces.

Then, for each of the prepared bleach solutions, the experiment proceeded as follows: a sample of about 100 g of lettuce was taken from the initial sample, cleaned once in tap water and then immersed in bleach; after acontact t emps of 5 min, a portion of this sample is removed from the solution and then rinsed again in cold water to remove chlorine residues; then 10 g of this rinsed sample is takenand then subjected to the analyses; after 10 min, another portion of the sample is removed from the solution, rinsed once and then analyzed; after 15 min, all the rest of the sample is removed from the solution, rinsed and then analyzed.

3. Methodology of microbiological analyses

For each of the analyzes carried out, a mother suspension is carried out by taking the ten grams (10 g) of lettuce in a Stomacher sachet to which have been added 90 ml of buffered peptonated water and then themixture is homogenized in a grinder. Then, from each parent suspension, successive decimal dilutions ranging from 10-2 to 10-5 (10-2, 10-3, 10-4 and 10-5) are carried out with the tryptone salt broth in test tubes and homogenized using the Vortex.

3.1. Enumeration of total flora

The total flora was researched and counted according to ISO 4833-1: 2013. One milliliter (1 ml) of the suspension and each dilution is taken and deposited in sterile Petri dishes and approximately 15 ml of Plate Count Agar agar has been added. After homogenizing the inoculum and the culture medium, the mixture is allowed to solidify and then a second layer of white agar of about 4 ml is added. After solidification, the boxes were turned over and incubated at 30°C for 72 hours. Then the reading is done by counting all the small white colonies and those at the head of the pin, only on the boxes that contain less than three hundred (300) colonies.

3.2. Dénombrement de *Escherichia coli* β-glucuronidase positive

The analysis was carried out according to iso 16649-2:2001. One milliliter of the stock suspension and each of the dilutions 10-2 and 10-3 is deposited in Petri dishes and then a volume of about 15 ml of tryptone Bile X-glucuronide (TBX) osel is poured into the box. After homogenization, the mixture is allowed to solidify. The boxes have turned over and incubated at 44 ° C for 24 hours and then the characteristic colonies (greenish blue colonies) are counted. Only boxes containing no more than one hundred and fifty (150) characteristic colonies were taken into account. Finally, the characteristic colonies have the object of identification thanks to the API 20E gallery.

3.3. Enumeration of coagulase-positive *Staphylococcus*

The research and enumeration of coagulase-positive staphylococci was carried out in accordance with ISO 6888-2: 2003. One milliliter of the mother suspension and each of the dilutions $^{10-2}$ and 10-3 is deposited in three Petri dishes and then a volume of about 15 ml of the Baird-Parker medium with the addition of rabbit plasma fibrinogen (Rabbit Plasma Fibrinogen) is added. After mixing the inoculum with the culture medium and allowing it to solidify, the cans turned over and incubated at 37 ° C for 24 hours. The reading is done by counting the gray-black colonies with light halo.

For each bacterial species enumerated, the load contained in 1g of sample was calculated by the following formula:

$$N(\text{UFC/g}) = \frac{n_1 + n_2}{1{,}1} \times \frac{1}{d_1}$$

where n_1 is the number of colonies counted at the 1st dilution (d_1) and n_2 is the number of colonies counted at the next dilution (d_2). The results are expressed in logarithm decimal.

RESULTS

1. Demographic parameters and frequency of lettuce consumption by participants

Of the 79 consumers surveyed, 50 come from the municipality of Abomey-Calavi and 29 from Cotonou. They are composed of 72 women and only 7 men with an average age of 31.3 (± 8.9) years. Respectively, 64.1% and 10.2% of consumers had the 1st and 2nd university cycle levels; 20.5% of them had the secondary level, only 1.4% had the primary level and 3.8% were out of school. Respectively, 10.1% and 34.2% of participants consume lettuce once a week and once a month while 3.8% consume it daily (Table I).

Table I. Frequency of consumption of lettuces by the 79 consumers.

Frequency of consumption	(%)
1 time/week	10,1
2 times/week	6,3
3 times/week	3,8
1 time/month	34,2
1 time/2 months	10,1
1 time/3 months	10,1
2 times/month	14
3 times/month	5,1
4 times/month	2,5

Every day	3,8

2. Practices for treating lettuce before they are consumed by participants

According to survey data, 50.6% of consumers surveyed use potassium permanganate to disinfect lettuces, 19% use bleach, 12.6% cook salt and 1.3% lemon (Figure 1). Some combine two or three products: 6.4% (05/79) treat lettuces once with potassium permanganate and then once with cooking salt; 2.5% (02/79) use bleach and then cooking salt; 2.5% combine potassium permanganate, bleach and cooking salt; only one consumer uses lemon, cooking salt and then potassium permanganate. 3.8% of consumers use other products namely vinegar (02/79) and potassium bicarbonate (01/79).

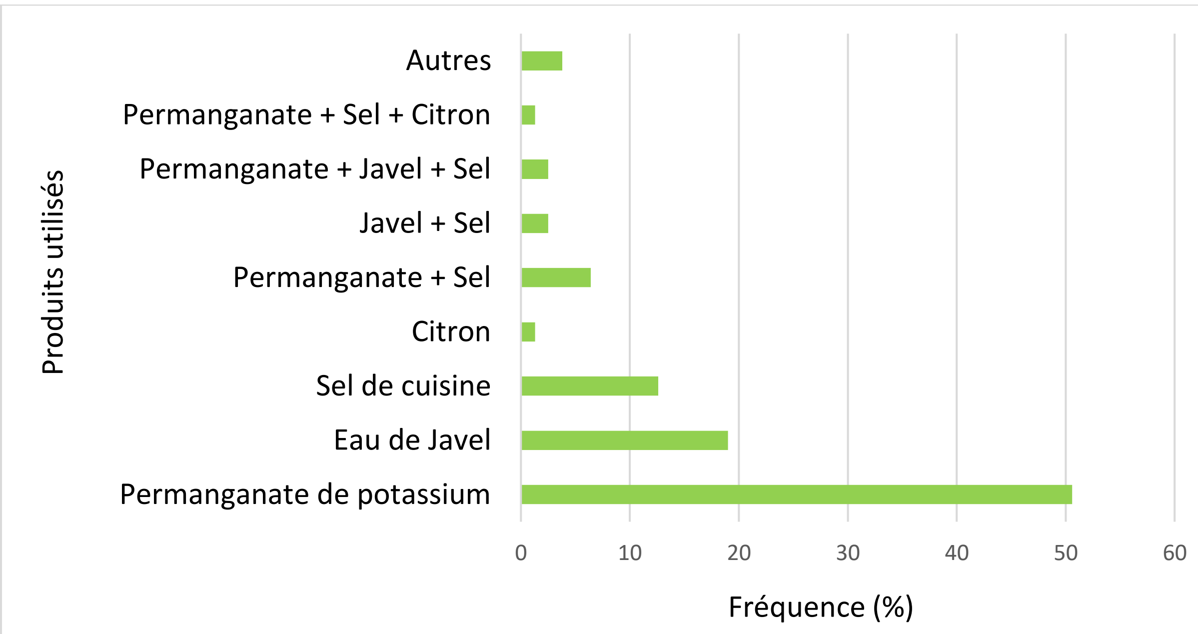

Figure 1. Products and combinations of products used by the 79 consumers for the disinfection of lettuce

In addition, 27.9% (22/79) of consumers clean lettuces with ordinary water before applying the disinfectant product(s); 11.4% (09/79) of them do not rinse lettuces after applying the disinfectant product(s). Not all participants were able to accurately say how much product and how much water they use to treat lettuce; in general, they carry out the dilutions intuitively according to the amount of lettuce to be consumed.

Between potassium permanganate and bleach which are the two products most used by consumers, we chose bleach for the conduct of laboratory experiments because bleach is a powerful disinfectant, available everywhere unlike potassium permanganate which is a detergent. In addition, chlorinated products are inexpensive and have a wide spectrum of activities against microbes, are effective at low temperatures and, in general, do not leave residues on surfaces (Massicotte, 2009).

3. Effect of bleach on the bacterial flora of lettuces

Table II. Evolution of the charges bactériennes (log UFC/g) on theitues after disinfection with bleach at different concentrations and according to the time.

		Total flora	*E. coli*	*Staph. coag+*
Uninfected lettuce		7,0	3,1	3,3
Disinfected lettuce				
Concentrations	**Contact time**			
0,12° chl	5 min	5,2	< 1	2,9
	10 min	4,5	< 1	2
	15 min	< 4	< 1	< 2
0,06° chl	5 min	5,5	1,3	3,0
	10 min	5,1	< 1	2,3
	15 min	4,6	< 1	< 2
0,024° chl	5 min	5,7	1,5	3,1
	10 min	5,3	< 1	2,9
	15 min	4,8	< 1	< 2

According to the results of the microbiological analyses of lettuces treated with bleach (Table I I), in general, we notice that the load of each bacterium sought decreases when the concentration of bleach increases and for the same concentration of bleach, the higher the contact time with lettuce, the more the load of bacteria decreases on them. The three concentrations of bleach tested reduced the total flora to a load of less than 6 log CFU/g on all treated lettuce. This result is satisfactory with regard to french (CNERNA) and Quebec (CECMA) standards which set the load of the total flora at less than 6.7 log UFC/g and 7 log CFU/g (Table III) respectively on raw plant products ready to be consumed.

Considering the specific flora of *Escherichia coli*, bleach at 0.12 ° chl eliminated almost all after only 5 minutes of contact with lettuces and it is after at least 10 minutes that this bacterial flora was almost completely eliminated at the concentration of 0.06 °chl. These results comply with French standards (Decree of 22 March 1993 and CNERNA) and the Québec standard.

Table III. Some microbiological criteria (expressed in log CFU/g) applicable to raw plant products ready for consumption.

	Total flora	*S. coagulase+*	*E. coli*
Fresh fruit salads			

Order of 22 March 1993[a] (n = 5)		< 1	< 1
Dessert standard[b] (n = 5)		2	
Products of IVth range*			
CNERNA[c] (n = 5)	< 6.7		< 2
CECMA[d] (n = 5)	7	2	1

[a] Order of 22 March 1993 on hygiene rules applicable to raw plants and plant preparations ready for human consumption (France).

[b] Internal standard for desserts (Grand Duchy of Luxembourg)

[c] National Centre for Studies and Recommendations on Nutrition and Diet (France).

[d] Committee on the Development of Microbiological Criteria in Foods (Quebec).

*Packaged products, raw, fresh, ready for consumption and having been peeled or cut

Sources: Guiraud (2003); Grand Duchy of Luxembourg (2015); CQIASA (2009)

With regard to the flora of *Staphylococcus* with coagulase positive, it should be noted that it is after at least 15 min that a residual flora less than 2 log CFU / g was obtained on the lettuce regardless of the concentration tested, which complies with the standards of Luxembourg (Standard of desserts) and Quebec according to which the number of *Staphylococcus* with coagulase positive must not exceed 2 log CFU / g. However, it is observed that overall, the reduction in the number of staphylococci was not significant enough compared to the initial number detected on raw lettuces.

DISCUSSION

The frequency of consumption of lettuces by the participants interviewed in our study shows that lettuce occupies a significant place in the diet of the populations of South Benin, hence the interest in being concerned with the health quality assurance of this market gardening speculation.

Taking into account normative and economic recommendations, the results of our study suggest that bleach at 0.06° chl for 15 min would be effective in eliminating substantially both *Escherichia coli* and coagulase-positive *Staphylococcus* flora which are germs harmful to human health. This finding confirms Weissinger et *al.* (2000) who reported that sodium hypochlorite (NaClO) at 200 ppm, i.e. at the concentration of 0.06° chl (1° chl = 3170 ppm), is widely used for vegetable disinfection. Therefore, Takeuchi and Frank (2000) reported that treatment with NaClO at 200 ppm for 5 min resulted in a reduction of 0.7 to 1.0 log CFU in the number of *Escherichia coli* O157: H7 on lettuces. As the results of Kondo et *al.* (2006), bleach at 0.06 ° chl did not cause the browning of lettuces in our experiments, the same is true for the other two concentrations tested.

Unlike *Escherichia coli*, none of the bleach concentrations tested in our study eliminated almost all of the coagulase-positive *Staphylococcus* flora. This ideal would probably have been achieved at higher concentrations of bleach for example at dilution to 1/50th (0.24° chl). However, high concentrations of bleach can be toxic to the body especially in children and people with fragile stomachs, ulcerative for example. In addition, knowing that bleach is a mixture of NaClO and sodium chloride (NaCl) and that *Staphylococcus aureus* tolerates high salt concentrations (Oumelkheir and Abderrahmane, 2007), this could be the basis for persistence, i.e. the significant non-reduction of staphylococci on lettuces at the different concentrations tested. This result confirms the fact that *Staphylococcus aureus* is often involved in disinfectant resistance mechanisms (GARRY, 2006). Kondo et *al.* (2006) reported that the combination of NaClO at 200 ppm and mild heat (50°C) for 1 min was more effective than NaClO alone in reducing native flora on lettuces without causing them to turn brown.

According to our survey, cooking salt (NaCl) is the third most used product by participants to disinfect lettuces. Since "*Staphylococcus aureus* is able to multiply in the presence of NaCl at concentrations of up to 20%" (GARRY, 2006), cooking salt would not be ideal for disinfecting lettuce. In addition, high concentrations of salt or too prolonged contact of salt with lettuces could lead to an imbalance in the organoleptic quality of lettuces including wilting of the leaves and the acquisition of a too salty taste thus making lettuce unfit for consumption.

Potassium permanganate ($KMnO_4$), widely used by populations in southern Benin, is not often cited in the literature among conventional disinfectants. Rather, this compound has chemical applications in the laboratory and in industry. But it is used as a stabilizer in the preparation of Dakin's solution which is an antiseptic based on sodium hypochlorite, intended to clean wounds and mucous membranes (Slama and Djemil, 2004). For these reasons, it would not be advisable to use it to disinfect vegetables and lettuce.

CONCLUSION AND OUTLOOK

It should be remembered that in order to avoid any risk of food poisoning, it is strongly recommended to disinfect lettuces before consuming them, for example by applying bleach at 0.06 ° chl for 15 minutes and rinse thoroughly. In perspective, we plan on the one hand, to continue laboratory analyses by testing higher concentrations of bleach in order to study the evolution of the load of staphylococci (check if they will be completely eliminated); on the other hand, to examine, for example, the evolution of the bacterial load, during storage, on disinfected lettuces.

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