Assessment of Modulatory Effectof Some Synthetic Food Dyes (Carmoisine and Tartrazine) on Gut Microbiota of Albino Rats

ABSTRACT

Background:Food additives are commonly used to improve the color, taste, aroma, texture, and nutritional quality of processed foods. **Aim**: The aim of this study was to determine the effects of the artificial food colors carmoisine and tartrazine on the intestinal microbiota of rats. **Study design**: An experimental design approach was chosen. Using a randomized control method, twenty (20) albino rats were divided into five groups of four (4) rats each. **Methods**: 0.1 g of each dye was weighed and dissolved in 0.9 ml of water, giving a total of 1 ml administered orally. After two (2) weeks of acute exposure, stool samples were collected aseptically. The plates were incubated at 37°C for 24 hours and examined for bacterial growth. Bacterial burden and prevalence were determined using conventional and molecular techniques. **Results**: The food dyes showed a significant ($p=0.0043$) reduction in the bacterial load of carmoisine-treated rats compared to the control. Although the other treatment groups had a greatly reduced bacterial load, there were no significant measurements in their means ($p<0.05$) compared to the control. The frequency and percentage occurrence of bacterial isolates from the sample revealed that *E. coli* was 74 (29%), *Staphylococcus aureus* was 68 (26%), *Micrococcus* species was 65 (25%) and *Proteus* species by 53 (20%). The result showed that *E. coli* had the highest number of bacteria. The before-exposure group of rats were observed to have the highest bacterial abundances ranging from 28 (41.2%) to 21 (39.6%).While the lowest occurrences were noted in carmoisine + tartrazine group, which ranges from 6 (8.1%) to 3 (5.7%).The result from the use of the molecular method identified the bacteria as *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas xianmensis,and P. aeruginosa*. **Conclusion**: This work has shown that artificial food dyes interact with the gut microbiota of rats by reducing them and may result in microbial dysbiosis.

Keywords:*Food dyes, gut microbiota,microbial dysbiosis, bacteria load*

1. INTRODUCTION

The gut microbiota plays an important role in improving the diversity of the human gene pool and actively contributes to the metabolism of xenobiotics. The term “human microbiota” includes many collections of microorganisms, such as bacteria, viruses, archaea and some unicellular eukaryotes [1]. The abundance of this microbiota shows significant variability and exceeds the number of genes present in the human genome[21]. Furthermore, it represents an exceptionally dense ecosystem, containing almost 1000 different species within the human population [2]. The dynamic relationship between the host and the gut microbiota has attracted considerable attention in the last decade[22]. While the essential role of the gut microbiome in human health is increasingly recognized, there is a need for more comprehensive understanding of this complex interaction [3]. The history of food coloring can be traced back to the ancient Egyptian and Roman civilizations, where people used a variety of plant substances such as saffron, flowers, carrots, mulberries, beets and other plant products to enhance the color of their foods. The use of dyes can be traced back even further to prehistoric times [4]. Subsequently, in the mid-19th century, people began replacing natural colors with synthetic alternatives. As a result, synthetic food azo dyes (-N=N-) have become widely used due to the increasing popularity of preserved and quick-prepared

foods.Furthermore, it should be noted that these colors lack any nutritional content, do not possess any discernible health advantages, and do not serve as preservatives [5]. Food presentation is altered to cater to the evolving preferences of customers since the visual appeal of food is deemed significant in the decision-making process of end consumers [6].

To increase the aesthetic appeal of food, various ingredients are included in food. According to established conventions of global researchers and the guidelines of the Codex Committee on Food Additives and Contaminants (CCFAC), the consumption of colorants is regulated by the concept of Acceptable Daily Intake (ADI) [7]. Certain dyes such as tartrazine, carmoisine, indigotin, sunset yellow and allura red are of synthetic origin, but others are derived from natural sources such as carotene and chlorophyll.

Approved color additives are intended for a variety of purposes in the areas of food, cosmetics and pharmaceuticals [8]. While most colored food additives share similarities. The Food and Drug Administration (FDA) regulatory description categorizes color additives into two distinct classes: natural color additives, which are exempt from certification, and synthetic color additives, which require certification. Research conducted in Asia and Africa has shown that the use of color additives such as tartrazine and sunset yellow exceeds the acceptable daily intake (ADI), particularly during celebratory occasions and weddings [8]. Consumption of carmoisine, tartrazine and sunset yellow in foods has increased significantly among consumers, especially youth, without proper regulations. Tartrazine mostly impacts the younger demographic due to their limited ability to regulate their dietary choices and heightened susceptibility to the allure of vibrant colors compared to adults [9].

Recently, studies have shown that there is a rapid increase in gut microbiome virulence, increased microbial resistance, and increased xenobiotic-induced resistance. One of the factors responsible for this virulence is the abnormal use of artificial food colors and herbal preparations [10, 11]. It is known that bacteria break down many substances by producing different metabolites through their metabolic activities. Some of the metabolites produced could be beneficial and others harmful, which is why it is necessary to study the effect of commonly used food colorings on rat intestinal bacteria. Attention is focused on tartrazine and carmoisine due to their widespread use in various beverages and confectionery products, primarily to improve visual appeal and stimulate appetite [12]. The clinical significance of these metabolites produced in the human body depends on the amount of food coloring consumed. The aim of this study was to determine the effect of the artificial food colors carmoisine and tartrazine on the intestinal flora of rats.

2. MATERIALS AND METHODS

2.1Study Animal and Dyes

Twenty albino rats were purchased from the University of Port Harcourt Animal Farm, Choba, Port Harcourt. They were transported in a ventilated wire cage to the animal house at the Department of Medical Laboratory Science, Rivers State University, NkpoluOroworukwo, Port Harcourt. Before starting treatment with the dyes, they were allowed to acclimatize for two weeks. Tartrazine and carmoisine food colorings were used to treat the rats. These dyes were purchased from Florio Colori, Gessate Itali.

2.2. Dosage of Food Dyes

The study extrapolated the oral dosage of tartrazine as 10 g/kg from the observed LD_{50} of 11.25 g/kgobtained in previous study conducted in our Departmental Laboratory [23]. Again, the utilized concentration of carmoisineused was 17.5 g/kg which was also observed that the LD_{50} was 22.5 g/kg [24]. These concentrations were taken from the oral dosages noted for these food dyes.

2.3Study Design

This was an experimental study in which a simple randomized method was used to group the ratsinto 5 groups with 4 rats in each group. Group A (control) given onlyfeed, group B: Carmoisin + feed, group C:

tartrazin + feed, group D: Carmoisin + Tartrazin + feed and group E: rat before exposure. Group E was used to normalize the bacterial load. The rats were fed daily with grown chicken and water. The group treated with carmoisin was treated orally treated with a daily intake of 1.0 ml of the dye, as was the Tartrazin group. Subsequently, 0.5 ml were used by each of the two dyes to treat the carmoisin and tartrazin group. The treatment was carried out for two weeks and the stool samples were collected aseptic in sterile universal bottles.

2.4Bacteriological Examination

A tenfold serial dilution was made on each sample as one 1 g of each stool sample was weighed and dissolved in 9ml of sterile normal saline and mixed thoroughly to give a one in ten ($1:10^{-1}$) dilution. From the dilutions, 1ml of each of the homogenates was transferred to another 9ml of the diluents to give $1:10^{-2}$ and this was further diluted to a one in three dilution $1:10^{-3}$. A 0.1ml of the 10^{-3} dilution from each of the serially diluted samples was cultured on the prepared and dried culture media plates which are Nutrient and MacConkey agar respectively. Spread homogenously with a glass spreader. The plates were incubated at 37^{O}C for 24 hours. The growth plates were examined macroscopically for different bacteria colonies. The different bacteria colonies were counted and recorded as bacteria colony forming unit per ml (cfu/ml).

2.5Determination of Total Viable Count

The bacteria load was determined using the spread plate method. 0.1ml from the dilution series was placed on the center of the surface of the agar plate, the sample was evenly spread over the surface of the agar using a sterile glass spreader. The plates were incubated at 37^{O}C for 24hr. Bacteria colonies present on each plate were counted and the various cultural characteristics were noted.

2.6Identification of Bacterial Isolates

The identification of the growth bacteria colonies was done macroscopically and microscopically. The gross morphology of the bacteria growth on plates were studied including their colours, sizes, shapes, slime, edges and so on. The identities of the isolated bacteria were certified using cultural characteristics and biochemical tests which include catalase test, coagulase test etc.

2.7Molecular Identification of bacteria

A volume of 5 ml of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) medium was subjected to centrifugation at a speed of 14,000 revolutions per minute (rpm) for a duration of three minutes. The cells were suspended in 500 µl of normal saline and subjected to heat treatment at a temperature of 95°C for a duration of 20 minutes. The bacterial solution, which had been heated, was then cooled using ice and subjected to centrifugation at a speed of 14,000 revolutions per minute for a duration of 3 minutes. The liquid portion containing the DNA was carefully moved to a microcentrifuge tube with a capacity of 1.5 ml and thereafter kept at a temperature of -20 degrees Celsius for further reactions.The quantification of the isolated genomic DNA was performed using the Nanodrop 1000 spectrophotometer.

2.7.116S rRNA Amplification

The 16s ribosomal RNA (rRNA) region of the rRNA genes of the isolates was amplified using the 27F primer (5'-AGAGTTTGATCMTGGCTCAG-3') and the 1492R primer (5'-CGGTTACCTTGTTACGACTT-3') on an ABI 9700 Applied Biosystems thermal cycler. The amplification was carried out in a final volume of 50 µlfor a total of 35 cycles. The PCR mixture consisted of the X2 Dream Taq Master mix provided by Inqaba, South Africa, which included Taq polymerase, DNTPs, and MgCl2. Additionally, the mixture comprised primers at a concentration of 0.4M and the extracted DNA as the template. The polymerase chain reaction conditions used in this study were as follows: The PCR protocol consisted of an initial denaturation step at 95ºC for a duration of 5 minutes. This was followed by a denaturation step at 95ºC

for a duration of 30 seconds. Subsequently, an annealing step was performed at 52ºC for 30 seconds. An extension step was then carried out at 72ºC for 30 seconds, and this whole cycle was repeated for a total of 35 cycles. Finally, a final extension step was conducted at 72ºC for 5 minutes. The result underwent resolution on a 1% agarose gel under an applied voltage of 120V for a duration of 15 minutes, and afterwards, it was observed using a UV transilluminator.

2.8Sequencing

The sequencing procedure was conducted utilising the BigDye Terminator kit on a 3510 ABI sequencer, which was provided by Inqaba Biotechnological located in Pretoria, South Africa. The sequencing process was conducted using a final volume of 10 µl, including the following components: 0.25 µl of BigDye® terminator v1.1/v3.1, 2.25 µl of 5x BigDye sequencing buffer, 10 µM Primer PCR primer, and 2-10 ng of PCR template per 100 base pairs. The experimental parameters for sequencing were as follows. The experimental protocol included subjecting the samples to a thermal cycling regimen consisting of 32 cycles. Each cycle consisted of a denaturation step at 96°C for a duration of 10 seconds, followed by an annealing step at 55°C for 5 seconds, and finally an extension step at 60°C for a duration of 4 minutes.

2.9Phylogenetic Analysis

The acquired sequences were subjected to editing using the bioinformatics process known as Trace edit. Additionally, comparable sequences were retrieved from the National Centre for Biotechnology Information (NCBI) database using the BLASTN tool. The alignment of these sequences was performed using the ClustalX software. Evolutionary history was deducted via the Neighbor-Joining approach as implemented in MEGA 6.0. The evolutionary history of the analysed taxa is represented by the bootstrap consensus tree, which was obtained from 500 replicates. The Jukes-Cantor technique was used to calculate evolutionary distances.

2.10Data Analyses

Statistical analysis was done using GraphPad Prism (version 8.02). Results were presented as Mean ±SD. Chi-square, One-way ANOVA, P-value of ≤0.05 were accepted as significant results (with turkey's post) were the tools used. DNA sequencing was carried out using a targeted resequencing design on the MiSeq platform (Illumina, San Diego, California).

3. RESULTS:

3.1 Bacterial Load

Figure 1 represents the normalized bacterial level present in the rat after removal of bacterial load prior to treatment with the food dyes. The food dyes showed asignificant ($p=0.0043$) reduction in the bacterial load of carmoisine-treated rats compared to the control. Although the other treatment groups showed highly reduced bacterial load, there was no significant ($p<0.05$) in the mean levels compared to the control.

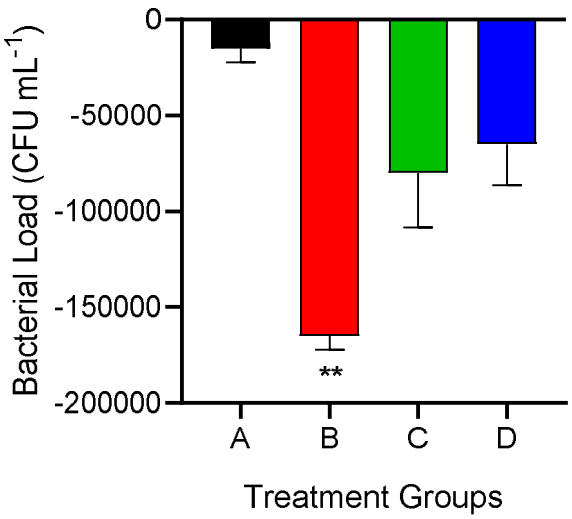

Figure 1. Total heterotrophic bacteria count.(Group A (control) food+water, group B: carmoisine + food + water, group C: tartrazine + food + water, group D: carmoisine + tartrazine + food + water and group E: rat before exposure. Group E was used to normalize the bacterial load)

3.2 Bacterial Prevalence

Table 1 shows the frequency and percentage occurrence of bacteria isolates. The highest percentage occurrence was observed in *Escherichia coli* 74 (29%). While the lowest frequency and percentage occurrence was observed in *Proteus species* 53 (20%).Table 2 shows the frequencies of bacteria in different treatment groups. It was observed that group E had the highest frequencies, which ranged from 28 (41.2%) to 21(39.6%). While the lowest frequencies and percentage occurrenceswere observed in group D, which ranges from 6 (8.1%) to 3 (5.7%).The overall chi-square comparison among the groups showed significant results (p=<.0001).

Table 1. Frequency occurrence of bacteria isolates

Bacteria Isolated	Frequency	Percentage Occurrence (%)
Staphylococcus species	68	26
Escherichia coli	74	29
Micrococcus species	65	25
Proteus species	53	20

Table 2. Frequency and Percentage occurrence of bacteria from different samples groups

Bacteria isolated	Group A (%)	Group B (%)	Group C (%)	Group D (%)	Group E (%)	Total
Staphylococcus species	20 (29.4)	10 (14.7)	6 (8.8)	4 (5.9)	28 (41.2)	68 (26.1)
Escherichia coli	23 (31.1)	11 (14.9)	8 (10.8)	6 (8.1)	26 (35.1)	74 (28.5)
Micrococcus species	19 (29.2)	9 (18.8)	7 (10.8)	5 (7.7)	25 (38.5)	65 (25.0)
Proteus species	17 (32.1)	8 (15.1)	4 (7.5)	3 (5.7)	21 (39.6)	53 (20.4)
Total	79 (30.4)	38 (14.6)	25 (9.6)	18 (6.9)	100 (38.5)	260 (100)
p-value			<.0001			
χ^2			122.9			

Legend: Group A (control) food + water, group B: carmoisine + food + water, group C: tartrazine + food + water, group D: carmoisine + tartrazine + food + water and group E: rat before exposure.

3.3 Bacteria Species Isolated

Some of the bacteria isolated using conventional techniques were subjected to molecular analysis in which the bacterial species were identified properly.The bacteria isolated using molecular techniques include *Escherichia coli*, *P. xiamenensis*, *P. aeruginosa* and *Klebsiella pneumoniae*.The 16s rRNA sequence obtained from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolate M1 showed a percentage similarity to other species at 100%.The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Pseudomonas, E. coli*and *Klebsiella* and revealed a closely relatedness to *P. aeruginosa*, *P. xiamenensis, Escherichia coli* and *K. pneumoniae*

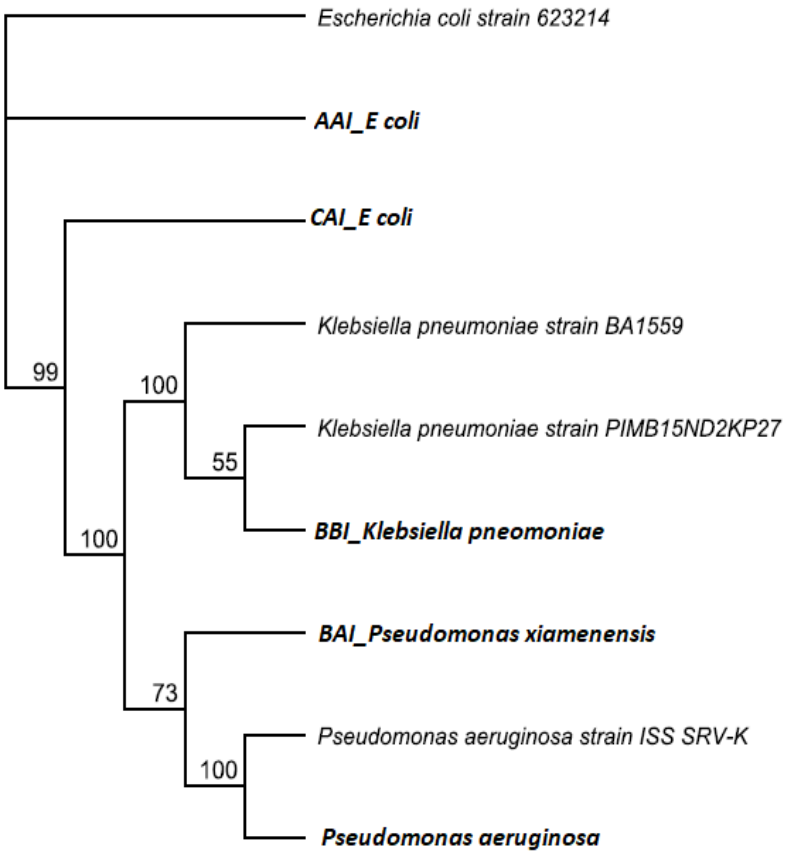

Figure 2. Phylogenetic tree showing the evolutionary distance between the bacterial isolates

4. DISCUSSION

This study investigated the effects of food dyes, carmoisine and tartrazine, on the gut microbiota of albino rats, focusing on bacterial load, diversity and species identification using conventional and molecular techniques. The results show significant changes in the microbial composition of the gut and highlight the effects of consumption of food dyes on microbial diversity and health. Figure 1 shows that the bacterial load was significantly reduced in rats treated with carmoisine compared to the control group. This reduction suggests that carmoisine may exert a bactericidal or bacteriostatic effect and alter the natural intestinal microbial ecosystem. Similar trends of reduced bacterial load were observed in other treatment groups, although these changes were not statistically significant compared to the control. These results are consistent with previous research indicating that synthetic food dyes can affect microbial viability and metabolic activity [13]. Other studies have shown that tartrazine exposure induced gut microbiota dysbiosis in juvenile crucian carp (*Carassius carassius*) [14]. In an in vitro experiment, *E. coli, Enterococcus faecium,Aerococcus viridans*, and

Bacillus cereus can decolorize Sunset Yellow, and tartrazine after 30 minutes of contact, which means those microbiomes have azoreductase activity [15].

The data in Table 1 highlights that *E. coli* is the most prevalent species with a frequency of 74 (29%), followed by *Proteus* species which had the lowest frequency and percentage of 53 (20%). The dominance of *E. coli* in the gut microbiota is consistent with its known role as a facultative anaerobe that thrives under diverse environmental conditions [16]. On the other hand, the lower prevalence of *Proteus* species could indicate selective suppression by food dyes or inherent competition between bacterial species under altered intestinal conditions [13].

The frequencies of bacterial isolates in different treatment groups, as shown in Table 2, show a marked variation in microbial composition. Group E had the highest frequencies of 28 (41.2%) to 21 (39.6%), suggesting that the specific treatment conditions favored the proliferation of certain bacterial species. In contrast, group D showed the lowest bacterial abundances (6 (8.1%) to 3 (5.7%), indicating significant suppression of microbial growth. These differences highlight the potential of food dyes to differentially influence microbial populations, possibly through mechanisms such as induction of oxidative stress or disruption of microbial homeostasis [13, 17].

Molecular analysis of bacterial isolates revealed the presence of *E. coli, P. xiamenensis, P. aeruginosa* and *K. pneumoniae*. 16S rRNA sequencing confirmed 100% similarity of the isolates to database entries, thereby confirming the reliability of molecular techniques in identifying bacterial species. Phylogenetic analysis using the Jukes-Cantor method further supported the evolutionary relationships between these species. These results are consistent with studies highlighting the robustness of 16S rRNA sequencing microbial taxonomy and evolutionary analysis [18].

The observed reductions in bacterial load and shifts in species prevalence highlight the potential risks of synthetic food dyes to gut health. Changes in the gut microbiota are associated with various health disorders, including inflammation, metabolic disorders and weakened immune responses [19]. The dominance of pathogenic species such as *P. aeruginosa* and *K. pneumoniae* in certain treatment groups raises concerns about dysbiosis that could predispose the host to opportunistic infections. Future studies on bacterial virulence such as biofilm production and antibiotic resistance should be investigated. Previous studies have observed that these virulence factors are induced by exposure of bacteria to xenobiotic substances [20]. This reported phenomenon requires further work in this study.

The phenomenon of microbial dysbiosis noted in the results of this study has clinical implications. A healthy gut microbial community assists in the breakdown of food for energy production [25]. Hence, imbalance in this community has been known to cause several kinds of diseases ranging from diabetes, metabolic syndromes, and obesity among others [26]. Further work needs to be done to establish that this relationship exists between food dyes and the aforementioned diseases.

There are potential impacts of confounding factors on gut microbiota prevalence. First, dietary factors have been shown to affect the distribution of patterns of gut microbiota as microbial communities use gut nutrients and obtain energy from digested carbohydrates and proteins in the host [27]. Our study exposed all rats to the same conditions which eliminates the dietary factor. However, the question of an interference between rat feed and the food dyes could arise. Some environmental variables such as temperature and pH have also been demonstrated to have affected both the level of bacteria and control virulence [28, 29]. This is because rats like other mammals have stomachs which contain a high acid environment and intestines that are slightly more alkaline. Individual variations in gut microbiota among rats before treatment could affect outcomes. This may be due to the immunological status and genetic factors of the host which may influence bacterial colonization [30].

To this end, future studies should focus on the chronic effect of these food dyes on animal models. Detailed investigation of the immunological variables of the exposed animals should be considered. Again, histological analyses of the effect of food dyes on animal studies need further exploration. In vitro, analysis of these food dyes on microbial communities can also be explored to depict a more controlled experiment.

5. CONCLUSION

Significantly reduced bacterial loads were observed in rats treated with carmoisine, indicating the ability of food dyes to act in a bacteriostatic or bactericidal fashion. There are changes in the prevalence of bacteria in all experimental groups, suggesting that different food dyes may have their pattern in microbial dysbiosis.

Disclaimer (Artificial intelligence)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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