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Phylogenetic analyses of bacteria associated with the processing of *iru* and *ogiri* condiments

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Running title: Bacterial succession in *iru* and *ogiri* processing

Significance and Impact of Study

Iru and *ogiri* are important condiments used for flavour enhancement in foods and serve as protein substitutes in diets among rural populations across West Africa. Consumption of these condiments is growing reinforcing the need to scale up their production. Production of these condiments includes spontaneous fermentation, which often leads to inconsistent product quality and unguaranteed safety. This study has demonstrated the bacterial succession in *iru* and *ogiri* processing and highlights species that could be selected and exploited for starter culture development. This study provides a starting point to produce quality and microbiologically safe *iru* and *ogiri* condiments.

Abstract

Analysis of the bacterial community dynamics during the production of traditional fermented condiments is important for food safety assessment, quality control and development of starter culture technology. In this study, bacteria isolated during the processing of *iru* and *ogiri*, two commonly consumed condiments in Nigeria, were characterised based on phylogenetic analyses of the bacterial 16S rRNA gene. A total of 227 isolates were obtained and clustered into 12 operational taxonomic units (OTUs) based on 97% 16S rRNA gene similarity. The OTUs spanned three phyla (*Firmicutes*, *Actinobacteria* and *Proteobacteria*), and nine genera: *Acinetobacter*, *Aerococcus*, *Bacillus*, *Enterococcus*, *Enterobacter*, *Lysinibacillus*, *Micrococcus*, *Proteus* and *Staphylococcus*.

OTUs closely related to species of *Bacillus* dominated the processing stages of both condiments. Although no single OTU occurred throughout *iru* processing stages, an OTU (mostly related to *B. safensis*) dominated the *ogiri* processing stages indicating potentials for the development of starter culture. However, other isolates such as those of *Enterococcus* spp. and *Lysinibacillus* spp. may be potential starters for *iru* fermentation. Presumptive foodborne pathogens were also detected at some stages of the condiments' processing, possibly due to poor hygienic practices.

Keywords: 16S rRNA gene; condiment; fermented foods; *iru*; *ogiri*; succession.

Introduction

Leguminous oilseeds such as those of African locust bean (*Parkia biglobosa*) and melon (*Colocynthis citrullus*) are widely fermented to produce condiments in many West African countries (Oguntoyinbo 2012). These condiments constitute a significant proportion of the protein content of diets in rural populations across West Africa (Achi 2005; Adedeji et al. 2017). In Nigeria, seeds of African locust bean and melon are fermented into *iru* and *ogiri* condiments respectively, though castor oil bean (*Ricinus communis*) is also used for *ogiri* production in some parts (Odunfa 1981, 1982, 1985).

Iru and *ogiri* are consumed mainly by rural dwellers where they serve as low-cost additives that enhance soup flavour (Odunfa and Oyewole 1986; Oyeyiola 1988). The processing steps for *iru* and *ogiri* production are very similar and generally involve sorting of the dried seeds, washing, boiling, dehulling, and fermentation at ambient temperature for about 72 hours and 120 hours for *iru* and *ogiri*, respectively (Odunfa 1982; Oyeyiola 1988). Processing of *iru* and *ogiri* from the raw materials is usually carried out on a small scale by rural dwellers in an uncontrolled environment and by spontaneous fermentation, which often

lead to inconsistent condiment quality as well as unguaranteed safety (with possible presence of pathogens) from one batch of production to another.

There are several reports available on the bacterial diversity in retailed *iru* (Odunfa and Oyewole 1986; Osho et al. 2009; Aderibigbe et al. 2011; Adewumi et al. 2013; Adedeji et al. 2017) and retailed *ogiri* (Odunfa 1982; Aniche et al. 1993; Akinyele and Oloruntoba 2013; Adedeji et al. 2017). However, besides the studies by Aderibigbe et al. (2011), Adewumi et al. (2013), and Adedeji et al. (2017), where molecular methods were applied in exploring the bacterial diversity in the retailed condiments, other diversity studies depended solely on classical microbiological methods of identification, which when compared to phylogenetic markers are known to be more prone to low taxonomic resolutions and are often misleading (Janda and Abbott 2007). Some of the previous studies presented a plethora of bacterial species including presumed fermenters and notable pathogens. Besides, only a few studies have attempted to explore the bacterial dynamics in *iru* (Odunfa 1981; Adelekan and Nwadiuto 2012) and *ogiri* (Barber et al. 1988; David and Aderibigbe 2010; Odibo et al. 2012), providing information that could be potentially useful for starter culture selection and controlled fermentation.

The application of molecular methods in exploring the diversity and dynamics of bacteria at major stages of processing of the two condiments are, however, very limited. A combined understanding of the bacterial diversity of the retailed products and bacterial succession during the fermentation of these condiments will provide information necessary for distinguishing actual fermenters from undesirable species and chance contaminants that might have been introduced during pre-processing, processing and post-processing stages of production. Since little is known about the bacterial diversity and successional patterns during *iru* and *ogiri* processing, this study employs 16S rRNA gene-based phylogenetic analysis to characterise bacteria associated with the processing of *iru* and *ogiri*. This will provide robust data on the bacterial successional patterns of both condiments for better food quality management.

Results and Discussion

A total of 227 bacterial isolates were recovered during the processing stages of *iru* (114 isolates) and *ogiri* (113 isolates). The 16S rRNA-based taxonomic diversity of these isolates is presented in Table 1.

Taxonomically, of the isolates obtained from the processing stages of *iru*, the majority belonged to the phylum Firmicutes (91.23%), while the remaining belonged to Proteobacteria (6.14%) and Actinobacteria (2.63%) (Fig. 1). The isolates were close relatives of species belonging to the genera *Aerococcus*, *Bacillus*, *Enterococcus*, *Lysinibacillus*, *Micrococcus* and *Proteus* (Fig. 1). Isolates obtained from the *ogiri* processing stages belonged to the phyla Firmicutes (67.49%), Actinobacteria (23.01%) and Proteobacteria (11.5%) (Fig. 2). At the genus taxa level, the isolates were close relatives of *Acinetobacter*, *Bacillus*, *Enterobacter*, *Enterococcus* and *Staphylococcus*. Species of *Bacillus* and *Enterococcus* were mostly associated with the African locust bean and its processing stages into *iru*, whilst only *Bacillus* species were common in *ogiri* processing stages. Additionally, *Lysinibacillus* was found during *iru* processing. The prevalence of *Bacillus* species in these condiments, as well as in other similar fermented bean condiments have been previously reported (Adewunmi et al. 2013; Ezeokoli et al. 2016a; Adedeji et al. 2017). Also, closely related species of *Acinetobacter*, *Enterococcus*, and *Staphylococcus* have been associated with these condiments and/or other similar fermented seed condiments (Nwosu and Ojimekwe 1993; Achi 2005; Ezeokoli et al. 2016a).

All 16S rRNA gene sequences obtained from both *iru* and *ogiri* processing stages clustered into 12 operational taxonomic units (OTUs), with eight OTUs obtained from the processing of *iru* and seven OTUs detected during *ogiri* processing stages (Table 1). Phylogenetic reconstruction revealed that the 16S rRNA gene sequences/OTUs belonged to

three major phylogenetic clades (Fig. 3) and that the clades were monophyletic, indicating close relatedness of sequences to one another and to sequences in the GenBank.

In this study, *Bacillus* species were dominant during *iru* and *ogiri* processing, with at least one species occurring at every stage of processing prior to fermentation of *iru*. This observation is similar to previous studies on traditionally fermented legume condiments such as *soy-daddawa* (Ezeokoli et al. 2016a) and *thua nao* (Chukeatirote et al. 2006). Species of *Bacillus* have also been previously reported to dominate in *iru* (Odunfa, 1981; Oyeyiola, 1988; Aderibigbe et al. 2011; Oguntinyinbo 2012; Adewumi et al. 2013; Adediji et al. 2017) and *ogiri* condiments (Odunfa 1982; Akinyele and Oloruntoba 2013; Adediji et al. 2017). The *Bacillus* species recovered in this study spanned three taxonomic groups, and the partial 16S rRNA gene sequencing was unable to completely resolve their exact taxonomies, hence whole genome sequencing of the *Bacillus* isolates is recommended for the final selection of starter cultures for both condiments.

The OTU groupings of bacterial species observed during the processing stages of *iru* and *ogiri* are presented in Fig. 4. Species of *Bacillus* (belonging to OTUs 1, 2 and 6) dominated *iru* processing, with OTU 1 and OTU 2 being associated with all stages, except in the river sand sediment-coated seed (Fig. 4a). On the other hand, OTUs 3 and 4 comprising species of *Enterococcus* and *Lysinibacillus*, respectively, were isolated from one or more processing stages, as well as in the fermented product. The source of these bacterial OTUs along the processing chain are likely due to these species being seed-borne, associated with the processing utensils, and/or introduced by the processor (Iwuoha and Eke 1996). The presence or isolation of specific OTUs (e.g. OTU 1 and OTU 2 during *iru* processing and OTU 5 during *ogiri* processing) at only certain stages may be due to several reasons. Firstly, this could be due to constraints imposed by the prevailing conditions (treatment) at specific stages and the differential abundance and adaptation of species to the processing conditions. Secondly, the microbial population in the seeds, processing materials and

utensils may predispose certain species to be more likely isolated (or missed) at certain stages. Thirdly, culture-based isolation of species are prone to the likelihood of missing out similar morphotypes (similar colony appearance) but phylogenetically distinct species and lastly, solid-state fermentations like *iru* and *ogiri* fermentations, are prone to substrate heterogeneity which may contribute to the underestimation of true species richness if representative samples are not analysed. However, attempts were made to collect a representative samples in the present study, and thus, the observed disappearance of species (e.g. OTU 2 during *iru* processing) at the fermentation stage are most likely not due to sample heterogeneity. It is now well documented that culture-based techniques for the study of microbial diversity are prone to underrepresentation of species (Ercolini 2003). Hence, future studies employing high-throughput culture-independent molecular analysis are required to obtain a deeper coverage of the bacterial diversity and dynamics during the processing of these condiments.

No specific OTUs was associated with all stages of *iru* processing (Fig. 4), suggesting inter-specific competition in several stages of the successional chain, which could lead to exclusion of some species in successive stages, creation of conducive environment for new colonizers, and induction of variable successional patterns in the species (Odunfa 1981; Odunfa and Oyewole 1986). Also, reasons stated earlier on the limitation of culture-dependent microbiological analysis may contribute to the observation of no specific OTUs at all stages of *iru* production. Furthermore, the variable bacterial successional patterns observed between pre-fermentation stages and fermented products could be due to variable responses of bacterial species to changes in pH and other biochemical parameters within the fermenting substrates (African locust bean and melon seeds) (Achi 2005). The fermentation of *iru* and *ogiri* are typical alkaline fermentations with characteristic rise in pH from near neutrality to alkalinity (Odunfa 1982; Achi 2005). Similarly, the pH recorded during the processing of the African locust bean seeds to *iru* ranged from 6.0 in the raw seeds to 6.43 after 24 h of fermentation. For the processing of *ogiri* from the

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melon seeds, the pH increased from 5.58 in the raw melon seeds, thru 5.89 after boiling, to 8.38 during fermentation. The pH values of the final products (*ogiri*) of the mashed and unmashed seeds were 7.82 and 7.64, respectively.

OTUs 5 and 8, which included potential pathogenic bacterial species *Proteus mirabilis* and *Aerococcus urinaeequi*, were only isolated during the stages prone to direct contact with the processor. No OTU was isolated from all stages of *iru* processing (Fig. 4a). During *ogiri* processing, *Bacillus* species (OTU 1) dominated the processing chain (Fig. 4b). *Bacillus safensis* was the dominant species associated with all the stages of *ogiri* production, except the fermentation stage of the unmashed (Batch B) seeds, where *B. altitudinis* was found to be dominant. As with *iru* processing, potentially pathogenic bacterial species belonging to OTUs 5, 7, 9, 10 and 11 were isolated mainly from the stages involving direct contact of the processor with the product (Fig. 4b and Table 1).

The recovery of *B. safensis* (OTU 1) and other species of *Bacillus* at all processing stages of *iru* processing and at almost all stages of *ogiri* processing, could be attributed in part to their ability to adapt to extremely harsh conditions and to out-compete other microorganisms (Cazorla et al. 2007; Kothari et al. 2013). Furthermore, the alkaline fermentation of the locust bean and melon substrates, which is often characterised by an increase in pH due to protein hydrolysis to produce amino acids, ammonia and peptides (Ouoba et al. 2003; Achi 2005), may have also favoured their proliferation than the other recovered species. In view of the successional data obtained, it is obvious that *B. safensis* and other species of *Bacillus* from *ogiri*, as well as the *Bacillus*, *Enterococcus* and *Lysinibacillus* genera from *iru* processing could play functional roles in the fermentation of the condiments. More efforts are, however, required to investigate the successional patterns of *iru* and *ogiri* processing using metagenomics tools which best elucidates community dynamics, and to study the functional roles of species within these frequently recovered genera.

The recovery of *Staphylococcus* species that belong to the same OTU only at the end of the processing of one batch of the fermented *ogiri* suggests possible contamination from leaf wrappings, fermentation materials and/or handlers (Achi 2005). Furthermore, the recovery of potential pathogens such as *Enterococcus faecium* and *P. mirabilis* in both fermented condiments, and the additional presence of *P. mirabilis* in some stages of processing where processors directly handle the intermediary products, raise concerns regarding the personal hygiene of processors and the hazards associated with the consumption of these condiments. It should also be noted that *P. mirabilis* was recovered in retailed *iru* and *ogiri* (Adedeji et al. 2017) suggesting that handling by processors is the major source of contamination of the condiments.

In conclusion, this study has provided data on the diverse bacteria populations associated with *iru* and *ogiri* processing. The study has further shown that species of *Bacillus* (*B. encimensis* and *B. safensis*), *Enterococcus* (*E. dispar*), and *Lysinibacillus* (*L. fusiformis* and *L. macroides*) may be the functional bacteria in *iru* processing while *B. safensis* may be the choice starter species for *ogiri* processing. There is also evidence that poor hygiene could affect the quality of the final products, and pose a public health threat to consumers. Thus, practical steps to improve the processing of the two condiments may include educating local processors on improvement of personal hygiene during processing such as use of gloves when direct contact with processing intermediate or final product is required, proper cleaning of processing equipment, and an exploration of the possibility of controlling the processing conditions. The latter is, however, possible when starter cultures have been carefully selected and developed.

Materials and Methods

Production of *iru* and *ogiri* condiments

African locust bean (*P. biglobosa*) and melon (*C. citrullus*) seeds purchased from a local market in Abeokuta, Nigeria, were processed into *iru* and *ogiri*, respectively. *Iru* was produced from the bean seeds by following the method commonly used by local processors in Abeokuta as shown in (see Fig. S1a in Supplementary Information). Briefly, the bean seeds were first sorted to remove defected seeds prior to washing in tap water. The washed seeds were boiled for 24 h and dehulled using a mechanical dehuller. Thereafter, dehulled seeds were coated with river sand sediment (locally called “*oloro*”) for 30 min, rinsed in tap water and boiled in brine for 2 h. After boiling, seeds were placed in a jute sack and allowed to ferment for 24 h at ambient temperature.

For *ogiri* production, melon seeds were processed as shown in Fig. S1b (in Supplementary Information). Briefly, shelled melon seeds were sorted to remove defective seeds, and then washed in tap water. The washed seeds were boiled for 6 h, dehulled using a mechanical dehuller, and then boiled for a further 2 h. Thereafter, the seeds were divided into two lots. Seeds in the first lot (Batch A) were mashed in a mortar prior to fermentation for 4 d (Asagbra et al. 2012), while seeds in the second lot (Batch B), as a modified method, were fermented prior to mashing (see Fig. S1b in Supplementary Information). The fermented products of each lot were then wrapped in banana leaves and dried by smoking (kept near a fire place) for 4 h prior to mashing into paste in a mortar.

Sampling during *iru* and *ogiri* processing

During *iru* processing, raw seeds, boiled seeds, dehulled seeds, river sand sediment-coated seeds (*oloro*), reboiled seeds, and fermented product (*iru*) were sampled, whilst the shelled melon seeds, washed seeds, boiled seeds, and fermented product (*ogiri*) of both

batches (mashed and unmashed) were sampled during *ogiri* production. The samples were collected into sterile plastic bags, stored at 4°C and analysed within 24 h.

Determination of pH of samples

For pH determination, sample homogenate (1:20 w/v in distilled water) were analysed using a pH meter (HANNA 2210, Woonsocket, RI, USA).

Microbiological analysis of samples from the production of condiments

Isolation

Bacteria were isolated from ten-fold dilutions of samples by the pour plate method. Nutrient agar (LAB M, Heywood Lancashire England) was used for the isolation of aerobic bacteria, MacConkey agar (LAB M, Heywood Lancashire England) for coliforms, and Mannitol salt agar (Oxoid, Basingstoke, Hampshire, England) for staphylococci. The inoculated culture media were incubated aerobically at 37°C for 48 h. Distinct colonies observed on the culture plates were successively streaked on fresh Nutrient agar, Mannitol salt agar or MacConkey agar plates to obtain pure cultures.

16S rRNA gene-based phylogenetic analyses of isolates

For phylogenetic analyses, genomic DNA was extracted from pure cultures of isolates by using the ZR fungal/bacterial DNA extraction kit (Zymo Research, California, USA) according to the manufacturer's instruction. Thereafter, the partial 16S rRNA gene was amplified using universal bacterial primers 27F and 1492R as previously described by Ezeokoli et al. (2016a). Amplicons were then purified and sequenced as previously described (Bamidele et al. 2017). Following sequencing, sequences were inspected and

manually edited using Chromas lite (v.2.1, Technelysium Pty Ltd) prior to aligning sequences against sequences in the EZBioCloud database (<http://www.ezbiocloud.net/>; Yoon et al. 2017). The 16S rRNA gene sequences obtained from this study are available in the GenBank under the accession numbers KY569461-KY569508. An operational taxonomic unit (OTU)-based taxonomic assignment of sequences was further performed by clustering sequences at 97% partial 16S rRNA gene similarity, using the mothur software (Schloss et al. 2009) as described by Ezeokoli et al. (2016b).

For phylogenetic analyses of isolates, representative sequences of each phylotype (based on the closest hits obtained from alignment on the EzBioCloud database) along with closely related sequences in the database GenBank were aligned using Clustal X (version 2.0) multiple sequence alignment program (Larkin et al. 2007). The multiple sequence alignment was manually edited for gaps and ambiguous alignment positions in MEGA7 software (Kumar et al. 2016). Thereafter, a neighbour-joining phylogenetic tree was constructed in MEGA7 as previously described by Adedeji et al. (2017).

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Conflict of Interest

The authors have no conflict of interests to declare.

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Table 1. Taxonomic affiliations of isolates obtained during the processing of *iru* and *ogiri* based on EZBioCloud database matches of partial 16S rRNA gene.

Processing stage	Lab ID	Closest hit on EzBioCloud	N ^a	OTU ^b	Similarity (%)	Accession number
<i>Iru</i> production						
Dried seeds	RS01	<i>Bacillus anthracis</i>	16	2	100	KY569461
	GS02	<i>Enterococcus dispar</i>	13	3	99.88	KY569462
Boiled seeds	BDS09	<i>Bacillus anthracis</i>	3	2	100	KY569468
	BDW05	<i>Bacillus encimensis</i>	4	6	100	KY569464
	BDS08	<i>Bacillus subtilis</i> subsp. <i>inaquosorum</i>	9	1	100	KY569467
	BDS07	<i>Enterococcus casseliflavus</i>	2	3	99.65	KY569466
	BDW06	<i>Lysinibacillus macroides</i>	9	4	100	KY569465
Dehulling	RW12-1	<i>Aerococcus urinaeequi</i>	10	8	100	KY569471
	DS10	<i>Bacillus altitudinis</i>	2	1	100	KY569469
	RW12-3	<i>Bacillus anthracis</i>	1	2	100	KY569473
	RW13	<i>Bacillus encimensis</i>	3	6	100	KY569474
	RW11	<i>Bacillus velezensis</i>	1	1	100	KY569470
	RDW13-2	<i>Enterococcus gallinarum</i>	1	3	100	KY569487
	RDW13-3	<i>Lysinibacillus macroides</i>	1	4	100	KY569486
	RW14	<i>Proteus mirabilis</i>	2	5	100	KY569475
River sand sediment-coated seeds	O23	<i>Bacillus safensis</i>	4	1	100	KY569484
	O24	<i>Lysinibacillus fusiformis</i>	3	4	99.89	KY569485
Reboiled seed	RDS16	<i>Bacillus anthracis</i>	13	2	100	KY569478
	RDS17	<i>Bacillus cytotoxicus</i>	4	2	100	KY569479
	RDW15-1	<i>Micrococcus endophyticus</i>	3	12	100	KY569476
Fermented products (<i>iru</i>)	12HR19	<i>Enterococcus faecium</i>	1	3	100	KY569481
	12HR18	<i>Lysinibacillus fusiformis</i>	4	4	99.87	KY569480
	24HR20	<i>Proteus mirabilis</i>	5	5	99.88	KY569482
<i>Ogiri</i> production						
Shelled melon seeds	CFD	<i>Bacillus safensis</i>	4	1	100	KY569489
	NPS	<i>Bacillus zhangzhouensis</i>	2	1	100	KY569488
	FA	<i>Enterobacter cloacae</i> subsp. <i>Cloacae</i>	2	7	100	KY569501
Washed seeds	NTS	<i>Bacillus safensis</i>	5	1	99.26	KY569506
	MPF	<i>Proteus mirabilis</i>	7	5	100	KY569508

Boiled seeds	MPS	<i>Acinetobacter variabilis</i>	3	10	100	KY569505
	NBM	<i>Bacillus safensis</i>	11	1	100	KY569507
Fermented product (Batch A <i>ogiri</i>)	COL	<i>Acinetobacter baumannii</i>	21	11	99.76	KY569491
	SBN	<i>Acinetobacter variabilis</i>	2	10	99.15	KY569494
	FB_1	<i>Bacillus altitudinis</i>	1	1	100	KY569499
	ME	<i>Bacillus safensis</i>	30	1	100	KY569492
	NMB	<i>Bacillus siamensis</i>	3	1	100	KY569497
	FA1	<i>Staphylococcus haemolyticus</i>	1	9	100	KY569504
	FP	<i>Staphylococcus schweitzeri</i>	4	9	99.76	KY569503
	MOK	<i>Bacillus altitudinis</i>	6	1	100	KY569493
Fermented product (Batch B <i>ogiri</i>)	NCT	<i>Enterobacter cloacae</i> subsp. <i>Cloacae</i>	1	7	99.88	KY569495
	FF	<i>Enterococcus faecium</i>	7	3	100	KY569502
	MPA	<i>Proteus mirabilis</i>	3	5	100	KY569498

^aNumber of isolates.

^bOperational taxonomic units (97% 16S rRNA gene similarity) number. OTU numbers are for combined sequences of *iru* and *ogiri* (See also Fig. 4).

^cAccession number of representative phylotype.

Figure Captions

Figure 1 16S rRNA gene sequence-based taxonomic distribution of isolates from *iru* processing. Percentages of isolates within bacterial phyla are shown as well as the relative percentage distribution of bacterial genera or species within each phylum.

Figure 2 16S rRNA gene sequence-based taxonomic distribution of isolates from *ogiri* processing. Percentages of isolates within bacterial phyla are shown as well as the relative percentage distribution of bacterial genera or species within each phylum.

Figure 3 Unrooted-Neighbour joining phylogenetic tree showing association of 16S rRNA gene sequences obtained from both *iru* and *ogiri* with sequences in the GenBank. Representative phylotypes from processing stages of each condiment were selected for phylogenetic reconstruction. Root tip labels with shaded triangles represent phylotypes from *iru* processing stages, while shaded circles represent phylotypes from *ogiri* processing stages. Accession numbers are in parenthesis. Bootstrap values (1000 replicates) <50% are not shown.

Figure 4 Operational taxonomic units (OTUs) (97% 16S rRNA gene similarity) associated with different stages of *iru* (a) and *ogiri* (b) condiment processing. OTU numbers for each processing point corresponds to the OTU numbers and taxonomic species listed against each condiment as described in Table 1.

Supplementary Figure Caption

Figure S1. Flowcharts for the production of *iru* (a) and *ogiri* (b).

Figure 1

Figure 2

Figure 3

Figure 4